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Pathogenesis of porcine reproductive and respiratory syndrome virus-induced maternal reproductive failure

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Pathogenesis of porcine reproductive and respiratory syndrome
virus-induced maternal reproductive failure

by

Kelly Milton Lager

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Major Professors: William L. Mengeling and Prem S. Paul

Iowa State University

Ames, Iowa

1998

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is a disease of swine that was first recognized in the late 1980's as epizootics of maternal reproductive failure and severe respiratory disease in young pigs. The etiology, the PRRS virus (PRRSV), was discovered in 1991 and is classified as a new member of the virus family Arteriviridae. Maternal reproductive failure attributed to PRRSV is characterized by increases in late-gestation abortions, early farrowings, weak pigs, stillborn pigs, and late and regular returns to estrus. Following natural exposure, it appears gilts and sows may develop a protective immune response to PRRSV.

This dissertation compiles studies beginning in 1992 that investigated the pathogenesis of PRRSV-induced maternal reproductive failure utilizing an experimental model that involved exposure of gilts and sows to PRRSV at various stages of gestation. Results indicated that PRRSV may have little detrimental effect on conception or embryonic development. However, as gestation progresses the incidence of transplacental infection increases and the fetus becomes more susceptible to the lethal effects of an intrauterine PRRSV infection. Homologous protective immunity was demonstrated that developed within 90 days after exposure to PRRSV and persisted for at least 600 days. In contrast, heterologous protective immunity may have limited cross-protection and duration, this may be due to antigenic relatedness of the challenge and vaccine virus. The cause of reproductive failure may be attributed to a virus-induced vasculitis that involves maternal and fetal tissues. The severity of vascular lesions is variable among fetuses within a litter and among gilts within the same treatment group. This may account for the diverse range of clinical signs attributed to PRRSV in the field. Additional studies demonstrated passively acquired immunity conferred limited protection to piglets, however, it did not prevent piglets from being infected and transmitting virus to contact controls. PRRSV is a primary swine pathogen that is capable of causing severe reproductive losses. Fortunately, a protective immune response can be induced with vaccine that may aid in the prevention and control of PRRS.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease of swine that was first recognized in North Carolina in 1987 (43). The emerging disease was characterized by two predominant clinical features; i.e., an acute onset of reproductive failure in the sow herd and respiratory disease in young pigs (69, 71, 80, 94, 182). Initially, the etiology was unknown and the new disease quickly became known by several names of which one, "Mystery Pig Disease," became the industry standard until 1991 at which time the etiology, the PRRS virus (PRRSV), was discovered (189) and the disease was renamed PRRS. Following the original epizootics, PRRS spread rapidly throughout the swine-dense regions of the United States and was reported in Canada (34) and Germany (17) in 1990; in The Netherlands (189), Spain (135), Belgium (183), Great Britain (191), Scotland, and France (5), in 1991; in Malta (115) and Denmark (15) in 1992; in Austria (115), Japan (156), Korea (78), and the Philippines in 1993 (115). Except for Australia (52), every major swine producing country in the world that has methodically tested for PRRS has been able to demonstrate its presence making PRRS a classic example of an emerging disease that apparently began as a few clinical cases in 1987 and within a decade became a significant world-wide problem for the swine industry.

The discovery of PRRSV and the fulfillment of Koch's postulates by Dutch scientists at the Central Veterinary Institute, Lelystad, The Netherlands in 1991 (189) provided investigators around the world the opportunity to begin unlocking the mysteries of PRRS. Based on the strengths of their respective laboratories, a number of research scientists initiated programs to ask and answer specific questions about the microbiology of the virus and the epizootiology, pathogenesis, immunology and vaccinology of the disease. An ongoing research program investigating the disease began at the National Animal Disease Center in the spring of 1992. A major objective of this program, which is the basis for the graduate studies compiled in this dissertation, has been elucidating the pathogenesis of the maternal reproductive failure component of PRRS.

Dissertation Organization

This dissertation begins with a general introduction followed by a literature review, seven manuscripts, a general conclusion, four appendices that compile unpublished data, and a list of references cited at the end. Six of the manuscripts have been published; "Pathogenesis of *in utero* infection in porcine fetuses with porcine reproductive and respiratory syndrome virus" in *The*

Canadian Journal of Veterinary Research, “Pulmonary lesions in fetuses exposed *in utero* to porcine reproductive and respiratory syndrome virus” in *The Journal of Veterinary Diagnostic Investigation*, “Gross and microscopic lesions in porcine fetuses infected with porcine reproductive and respiratory syndrome virus” in *The Journal of Veterinary Diagnostic Investigation*, “Effect of post-coital intrauterine inoculation of porcine reproductive and respiratory syndrome virus on conception in gilts” in *The Veterinary Record*, “Homologous challenge of porcine reproductive and respiratory syndrome virus immunity in pregnant swine” in *Veterinary Microbiology*, and “Duration of homologous porcine reproductive and respiratory syndrome virus immunity in pregnant swine” in *Veterinary Microbiology*. The final manuscript, “Evaluation of homologous and heterologous immune responses in gilts with antigenically distinct porcine reproductive and respiratory syndrome virus isolates” has been submitted to the *American Journal of Veterinary Research*.

Kelly Lager was the principal investigator of each of the manuscripts. William L. Mengeling, Susan L. Brockmeier, Mark R. Ackermann, and Patrick G. Halbur contributed to the design and execution of the respective manuscripts. William L. Mengeling and Prem S. Paul were co-major professors who provided advice and counsel for the course of research.

CHAPTER 2. LITERATURE REVIEW

The literature review is divided into six sections. The first section is an anecdotal history of the disease prior to the discovery of the etiology followed by sections discussing the current state of knowledge on the etiology, epizootiology, pathogenesis, diagnosis, and prophylaxis of the disease.

History

Porcine reproductive and respiratory syndrome was first recognized as sporadic epizootics of maternal reproductive failure and respiratory disease in young pigs (69, 71, 80, 94, 182). Initially the etiology was unknown; however, based on observation it was thought to be infectious due to the temporal aspect of the onset of clinical signs in a sow herd and to the onset of clinical signs within a recipient herd into which recently affected pigs had been moved. During the late 1980's a number of putative etiologies were proposed as the cause of PRRS but each one was ruled out based on either a lack of fulfilling Koch's postulates (*L. bratislava*, *C. psittaci*, atypical influenza virus, fumonisin toxin) or the infectious agent was not associated with PRRS epizootics although it was capable of causing reproductive losses under experimental conditions (encephalomyocarditis virus). The viral etiology of PRRS was discovered in 1991 by Dutch scientists and named the Lelystad virus after their laboratory, the Central Veterinary Institute in Lelystad, The Netherlands (189). The clinical presentation of a PRRS epizootic was often complicated by enzootic infections resulting in a myriad of clinical signs that were attributed to PRRS. For the sake of clarity, the following discussion will concentrate on the salient clinical signs believed due to PRRSV that would be observed during a PRRS epizootic in a typical, farrow to finish production system involving at least 200 sows or more.

The reproductive failure associated with the epizootics was characterized by the following: 1) abortions that were reported during all stages of gestation, however, the vast majority occurred during the last 20 days of gestation and consisted of "fresh" fetuses, i.e., they were relatively free of autolysis; 2) early farrowings defined as the delivery of weak and stillborn pigs from gestation day (GD) 108 to 112 and a significant increase in the incidence of weak and stillborn pigs at term; 3) the onset of litters containing weak and stillborn pigs was followed by litters containing dead fetuses that were in varying stages of mummification; and 4) an increased incidence in returns to estrus and delayed returns to estrus (69, 71, 80, 94, 182).

Frequently, gilts and sows were clinically affected with anorexia, pyrexia, and listlessness prior to the onset of reproductive failure (69, 71, 80, 94, 182). Usually the illness was transient, however, it could last for a few days and possibly a few weeks resulting in loss of condition. Pyrexia usually

ranged from 40 to 41 C with a duration of 4 to 7 days. Rarely, gilts and sows in North America developed a cutaneous discoloration resulting in a cyanotic appearance to the extremities referred to as “Blue Ear” in Europe due to the dramatic discoloration of the ears. This phenomenon was usually transient and would dissipate within hours or a couple of days. In Europe, for reasons that are not understood, Blue Ear apparently affected a larger proportion of swine than in North America. The onset of clinical signs in the sow herd occurred in a “rolling” pattern, i.e., one day a few animals were affected followed by more on the next day and so on. The onset of reproductive failure occurred in a similar pattern with a few abortions or affected litters on one day followed by more and more on subsequent days. The incidence of illness and reproductive failure appeared to “jump around” in the breeding/gestation barn with some gilts and sows clinically unaffected during the epizootic. This acute phase usually lasted 4 to 6 weeks and was followed by a convalescent phase that had a high incidence of litters containing decreased numbers of live pigs with the remainder of the affected litter consisting of dead and mummifying fetuses. Eventually (3 to 6 months) most herds would approach pre-PRRS epizootic production levels; however, some herds suffered an enzootic form of PRRS that could persist for extended periods of time.

Numerous case reports have described the clinical effect of PRRS on the sow herd, however, few have calculated the losses during an epizootic. Abortion rates have been reported at 0.5 to 2% (80), 3 to 4% (94) and 5% (182). Mortality rates for the sow herd have been reported at 3% (182) and 3 to 4% (94). These incidence rates are assumed to mean percent of the herd affected during the epizootic and not an increase in the annual rates for the herd. The incidence of sick sows and affected litters during an epizootic have not been reported in North America. However, it is assumed that the morbidity rates were high because of the significant losses reported in herds within a short time, e.g., stillbirths ranging from 20 to 70%, (80, 94) and preweaning mortality rates ranging from 37 to 83% (80, 94, 123) reduced the average litter size at weaning to as few as 1 to 3 pigs per litter for several months. In general, the clinical presentation of PRRS epizootics throughout the world has remained similar (73, 131) with only slight variations being reported for the incidence rates of abortions, sow mortality, and piglet mortality. These minor differences may reflect actual differences in virulence or just biologic variation due to many variables.

Although boars appeared to be clinically affected at a lesser incidence than sows, they still developed anorexia, pyrexia, and listlessness (42, 49, 73, 168) which in some cases, was associated with poor breeding performance and minimal, temporary decreases in semen quality that developed 1 to 10 weeks after the acute infection (49). Circumstantial evidence suggested PRRS might be

transmitted with semen collected from acutely infected boars prompting much speculation that boars could shed an infectious agent in semen and the transportation of contaminated semen was responsible in part for the rapid spread of PRRS (48, 147).

The respiratory component of PRRS was characterized by dyspnea with a typical forced abdominal breathing in young pigs described as “thumping” (30, 80, 94). Usually this was most pronounced in weak-born pigs in the farrowing house where preweaning morbidity and mortality could reach 100 % and 80%, respectively (80, 94, 123). Pigs that survived were less affected in the nursery with thumping, however, a high mortality rate continued that was attributed to endemic bacterial diseases (80, 94). Growth performance in the nursery was severely depressed, usually throughout the finishing phase (80, 94). In a farrow to finish herd the onset of respiratory disease usually trailed the onset of reproductive failure although clinical signs were sometimes recognized in the nursery and grower prior to the epizootic of reproductive failure. The recovery from respiratory disease would usually trail the recovery of the sow herd, however, some farms with continuous flow production involving multiple source pigs appeared unable to clear the respiratory disease from the herd. In these herds reproductive losses were limited to incoming replacement gilts (37, 39). In most farms the sow herd eventually regained normal pig production despite apparent circulation of the agent in the nursery and grow/finish units. These observations implied the sow herd had developed immunity against the endemic PRRS agent which was circulating in the production units due to the continuous flow of naive piglets from the farrowing house (162, 163).

Dramatic economic losses were attributed to PRRS with loss estimates ranging up to \$236 per sow in inventory (139) and \$18 per pig space (121). Regional losses could be severe as reported in one survey which estimated losses at 10.6 million dollars for 250 affected farms within a 50 mile radius of an Iowa slaughter plant (4). Chronic losses that have followed these initial epizootics were difficult to fully evaluate. In countries that normally exported swine and pork products, significant losses due to trade barriers were experienced following the identification of PRRS within their borders, however, these barriers have eased somewhat since PRRS has essentially become a ubiquitous disease worldwide.

The spectrum of maternal reproductive failure just described was typically reported in larger swine farms that had frequent or continuous farrowing schedules resulting in a large number of pregnant swine housed together at different stages of gestation. The clinical signs reported on these farms were compatible with an infectious agent capable of infecting the dam and causing either direct reproductive failure, i.e., abortion or failure to conceive, or indirect reproductive failure, i.e.,

transplacental infection resulting in weak pigs, stillborn pigs, and fetal death. Usually, the classic spectrum of maternal reproductive failure observed on larger farms was not reported on smaller farms where most of the sow herd would farrow in a group at one time. When clinical signs were noticed they were usually limited, e.g., reproductive failure might be recognized as mostly abortions, or as mostly dead and mummifying fetuses, or an increase in returns to estrus. This scenario of a limited set of clinical signs in smaller herds is also compatible with an infectious agent capable of infecting swine and causing reproductive failure since most of the sows in small herds are in the same phase of the breeding cycle and the extent of clinical signs may be dependent on when they are infected. For example, infection of sows prior to breeding may produce few clinical signs with only a slight depression in fertility which could be easily missed or misinterpreted.

Prior to the discovery of PRRSV the etiologic agent was thought to be able to infect sows and produce illness within a few days and cause transplacental infection resulting in abortions or the expulsion of weak and stillborn pigs and dead and mummified fetuses. The clinical effect on fetuses was considered dependent on how long and at what stage of gestation the dam became infected. For example, infection of the dam near the time of parturition could result in the delivery of weak and stillborn pigs while infection of the dam a few weeks earlier in gestation could result in a lethal fetal infection. Moreover, the mystery agent (PRRSV) was believed to affect conception and the implantation of embryos resulting in early gestation losses or production of small litters. Interestingly, evidence for mid-gestation (approximately 38 to 76 days of gestation) reproductive failure, i.e., abortions during this stage of gestation or the observance of small mummies at parturition indicating fetal death during this stage of gestation, was seldom recorded in early reports. This could be due to the fact that mid-gestation reproductive failure was uncommon when compared to late-gestation, or it was as frequent but underreported because it was not as dramatic as late-gestation reproductive failure. Another possibility involved some unique aspect in the pathogenesis of this agent that resulted in more late-gestation than mid- or early-gestation reproductive failure despite sows being infected at all stages of gestation.

Diagnostic investigations into PRRS usually revealed little, except for the fact that known pathogens could not be identified and no consistent lesion of diagnostic value was associated with the reproductive failure. Gross and microscopic lung lesions indicating an interstitial pneumonia suggestive of a viral etiology were found in affected pigs, gilts and sows (30, 71, 204). The lesions were consistent among herds with high health status that experienced PRRS. However, in low health status herds the lesions were obscured by the development of bacterial infections which would

exacerbate the respiratory disease resulting in high mortality rates. The bacterial infections were considered secondary to the putative PRRS agent fueling the speculation that this agent was immunosuppressive and predisposed swine to enzootic infections (73, 80, 94).

The etiology of PRRS was believed to be a virus that was probably transmitted animal-to-animal and possibly by way of fresh semen. These modes of transmission, however, did not necessarily account for the rapid spread of PRRS throughout swine dense regions of Western Europe. During the Western European epizootics in the winter of 1990/1991, the climatic conditions were hypothetically ideal for aerosol spread of virus. This led to much speculation about the PRRS agent being aerosolized over considerable distances (42, 82), a possible explanation for the rapid spread of virus despite quarantine procedures.

Etiology

Porcine reproductive and respiratory syndrome virus was discovered by Dutch scientists who cultured it on porcine alveolar macrophages (189). This PRRSV isolate, known as the Lelystad virus, has become the European PRRSV prototype. In 1992, the North American PRRSV prototype, known as ATCC VR-2332, was discovered (30). It had been passaged twice through gnotobiotic pigs before it was eventually cultured on a proprietary continuous cell line of simian origin known as CL 2621 (11). A permissive clone of the MA-104 cell line, a simian kidney cell line, has been developed (81) and is widely used by itself or in conjunction with alveolar macrophages for diagnostic and research purposes. PRRSV has a single strand positive sense 15.1 kb polyadenylated RNA genome consisting of 8 ORFs enclosed within a cubical nucleocapsid and a lipoprotein envelope with an approximate size of 50 to 65 nm (11, 31, 97, 117). The virus is classified as a member of the Arteriviridae family which includes lactate dehydrogenase-elevating virus of mice (LDV), simian hemorrhagic fever virus (SHFV), and equine arteritis virus (EAV) (134). All of these viruses share a similar replication strategy, morphology, and host cell tropism (cells of the phagocytic lineage). Moreover, they can establish long-term or persistent infections in the host. The Arteriviridae are classified in the new order Nidovirales, whose members (Arteriviridae, Coronaviridae, and Toroviridae) share a common replication mechanism, i.e., replication via a nested set of mRNA (20, 31, 117). The entire genome of Lelystad virus has been sequenced (117). Approximately 80% of the 5' end of the genome encompasses what are believed to be the replicase genes ORFs 1a and 1b that are expressed by a putative ribosomal frameshift mechanism (31, 117, 134). Genetic analysis of this region has demonstrated the predicted Lelystad virus RNA polymerase

has functional domains homologous to the polymerase of EAV and members of the Coronaviridae and Toroviridae, thus substantiating an evolutionary relationship among these virus families (117). ORFs 2 to 7 form a 3' coterminal nested set of subgenomic RNAs that all have a common 5' leader sequence. Studies have demonstrated that ORFs 2 to 5 encode N-glycosylated structural proteins GP2, GP3, GP4, and GP5, respectively and ORFs 6 and 7 encode nonglycosylated proteins forming the integral membrane protein (M) and the nucleocapsid protein (N), respectively (3, 8, 31, 83, 98, 99, 102, 103, 117, 118, 119, 122, 125, 154). The physicochemical properties of these proteins have been summarized in Table 1A.

Genetic analysis of ORFs 2 to 7 for a number of PRRSV isolates have been completed and are summarized in Table 1B. In general, a surprising amount of genetic variability has been found among PRRSV isolates, this is compatible with RNA viruses lacking proofreading mechanisms. However, two distinct genotypes have been found, one in Europe, represented by the Lelystad virus and one in North America represented by ATCC VR-2332 (79, 103). Based on amino acid identity, the similarity of European isolates compared to Lelystad virus range from 88 to 100% depending on the ORF analyzed and when the isolate was collected (45, 165). In North America, the similarity of North American isolates compared to ATCC VR-2332 ranged from 88 to 98% and the similarities between North American and Japanese isolates when compared to Lelystad virus were 52 to 81%, again depending on which ORF was analyzed (3, 50, 83, 98, 99, 102, 103, 104, 122, 125, 154). As would be expected there were variable regions and conserved regions within the ORFs that have been demonstrated not only by genetic analysis but by monoclonal antibodies.

Monoclonal antibodies have been produced against the following viral proteins; GP3, GP4, GP5, M and N (36, 46, 95, 126, 133, 148, 180, 192). Although most studies have been limited in scope, conserved and divergent epitopes have been demonstrated between North American and European PRRSV isolates. The SDOW-17 monoclonal antibody raised against ATCC VR-2332 reacts with a highly conserved conformational epitope on the N protein that is believed to exist on almost all PRRSV isolates (126). Likewise, monoclonal antibodies produced against the N protein of several European virus isolates will also react with North American isolates (148, 192). Competitive binding assays have not been completed to clarify if these monoclonal antibodies are directed against the same domain or not. Preliminary monoclonal antibody mapping studies have revealed at least 2 domains on the N (46, 148) and GP5 proteins (133). There may be at least two domains on the GP3 protein; one appears to be conserved between North American and European viruses (192) while the other one may be highly variable (46). Monoclonal antibodies directed against GP4, GP5 and M

Table 1: Comparison of physicochemical characteristics and amino acid composition of Lelystad viral proteins with different PRRSV isolates.

1A. Predicted Molecular Mass (kd) of specific proteins of PRRSV isolates from around the world.

	A	B	C	D	E	F	G	H	I
ORF 2	GP2	2	28.4		29.4				29.5
ORF 3	GP3	7	30.6	29.0	29.0				28.7
ORF 4	GP4	4	20.0	19.6	19.5				19.5
ORF 5	GP5	2	22.4	22.4	22.4	22.2	26-30	22.2	
ORF 6	M	2	18.9	19.1	19.0	19.1	19.0	19.1	
ORF 7	N	1	13.8	13.6	13.5	13.6	15.0	13.6	

A-Tentative name of ORF gene product. B- Predicted glycosylation sites, Meulenberg, et al. 1993; Conzelman, et al. 1993. C-Meulenberg, et al. 1993; Conzelman, et al. 1993. D-Mardassi, et al. 1995. E-Murtaugh, et al. 1995. F-Saito, et al. 1996. G-Bautista, et al. 1996. H-Meng, et al. 1994. I-Morozov, et al., 1995.

1B Percent amino acid sequence identity between Lelystad virus and different PRRSV isolates from around the world.

ORF	A	B	C	D	E	F	G	H	I	J	K	L
2	63			58				57-61				
3	58		54	56				55-56	93-99			
4	68		68	67				65-67		64	64	
5	59	54	52 (90) ^a		55	88		51-55				51-55 (89-94)
6	78	78	81 (96)		81		78-81				78-80 (94-98)	
7	65	58	59 (96)		61	95-100	57-59		100		58-61 (90-95)	

()^a = percent amino acid sequence identity with North American prototype isolate ATCC VR-2332. A- Murtaugh, et al. 1995. B-Meng, et al., 1994. C- Mardassi, et al. 1995. D-Morozov, et al. 1995. E-Saito, et al., 1996. F-Suarez, et al. 1996; compared 14 European PRRSV isolates with Lelystad virus. G-Meng, et al., 1995; compared 5 US PRRSV isolates with Lelystad virus. H-Meng, et al., 1995b; compared 5 US PRRSV isolates with Lelystad virus. I-Drew, et al. 1997; compared 7 British PRRSV isolates with Lelystad virus. J-Kwang, et al., 1994. K-Gagon, et al., 1998; compared 15 Canadian PRRSV isolates. L-Andreyev, et al., 1997; compared 20 North American PRRSV isolates with Lelystad virus.

have not detected any epitope that is conserved between the North American and European isolates (36, 133, 180, 192). *In vitro* virus neutralization has been demonstrated with monoclonal antibodies against GP4 (180) and GP5 (133), however, the *in vivo* significance of these observations is not known.

Genetic differences between North American and European PRRSV isolates suggest they may have had a common ancestor from which they both derived sometime prior to the onset of clinical disease that was first recognized in the late 1980s in North America and in 1990 in Western Europe (79, 125). Although the origin of PRRSV is unknown genetic comparisons between it and other members of the Arteriviridae suggest PRRSV may be more closely related to LDV than EAV or SHFV (103, 125). This has led to much speculation that PRRSV and LDV arose from a common ancestor or that LDV may have adapted to swine and eventually gained virulence in this new population. Whether this is true or not it appears PRRSV began circulating in swine on the North American continent during the early 1980's based on retrospective serology studies identifying PRRSV-specific antibody in serum collected in Canada in 1979 (18) and in Iowa in 1986 (207).

Epizootiology

The infectious etiology of PRRS was confirmed with the reproduction of maternal reproductive failure in 1991 (175) and with reproduction of the respiratory disease in 1992 (30). Given the ability to culture and characterize PRRSV, scientists began to develop tools to answer questions about the epidemiology of this new disease that was systematically spreading around the world producing great anxiety and economic loss for the swine industry. Perhaps the most important questions to address first concerned the transmission of PRRSV that field observations had suggested could occur by movement of infected swine (48, 135), use of semen from infected boars (147), and by aerosol transmission (42, 82). A number of studies have been completed addressing factors associated with transmission of PRRSV that will be reviewed in the following manner: evaluating the susceptibility of the host, viral shedding, vectors, and transmission by contact, semen, and aerosol. Young and adult swine have been experimentally infected with PRRSV by aerosol, oronasal, intramuscular, intraperitoneal, and intravenous routes. Gilts have been infected by way of PRRSV-spiked semen (145, 173), and semen collected from boars acutely infected with PRRSV (171, 173). Young swine have been very susceptible to a PRRSV infection, i.e., pigs have been infected intramuscularly with as little as 10 tissue culture infectious dose₅₀ (TCID₅₀) virus particles (207). Adult swine are considered more resistant to infection, although this hypothesis has not been fully tested.

Experimentally, adult swine have been infected with as few as 100 TCID₅₀ virus particles administered intramuscularly (unpublished observations, Kelly Lager).

A viremia detectable by conventional virus isolation techniques has developed in swine of all ages following infection with PRRSV. In the case of young pigs, viremia has been detected up to 6 weeks following experimental infection (47, 149), in congenitally infected pigs up to 8 weeks post-partum, (107) and in the case of adult swine, usually 1 to 2 weeks after infection (107). In pigs virus has generally been detected for at least several weeks longer in bronchoalveolar lavage fluids than in serum (113). Polymerase chain reaction (PCR) assays have been used to detect PRRSV nucleic acid and presumably infectious virus in a variety of tissues. This methodology was shown in several studies to be more sensitive than virus isolation; one of which involved 2 congenitally infected pigs from which PRRSV was detected in serum for 7 weeks post-partum by virus isolation and PRRSV nucleic acid was detected by PCR in white blood cells for 210 days post-partum (10).

The frequency and duration of detectable virus shedding has been variable in experimentally infected swine. PRRSV was isolated occasionally from urine, feces, saliva, nasal swabs, and mammary secretions (149, 184, 195, 198). However, in a separate study virus was not isolated from feces following experimental infection, although it was isolated intermittently from serum and throat swabs for 21 and 157 days, respectively (195). Although it may be intermittent, swine have the potential to shed virus from several routes (nasal secretions, saliva, urine, feces, and semen[reviewed later]) for extended periods of time which emphasizes the important role of contact transmission and carrier swine in the epizootiology of PRRS.

The role of biological and mechanical vectors has been investigated. Mice are the natural host for LDV, a virus that has genetic and biological properties similar to PRRSV (116, 134). This has led to speculation that LDV may be an ancestor of PRRSV (31, 116) and that mice could serve as a potential interepizootic reservoir for PRRSV. This hypothesis was not supported by a study that concluded rats and mice (field and laboratory species) did not support PRRSV replication (72). A hypothesis suggesting cattle as a potential interepizootic reservoir for PRRSV was not supported by a preliminary study in which calves were oronasally exposed to PRRSV. The calves did not appear to replicate virus nor develop PRRSV-specific antibody following inoculation (Kelly Lager unpublished observations). To date, there appears to be no evidence for mammals other than swine acting as an interepizootic reservoir of PRRSV. However, a study testing the susceptibility of avian species to PRRSV infection indicated that at least some species of birds (Mallard ducks, guinea fowl, and chickens) can support replication of PRRSV and shed virus in feces (206). Moreover, the duck

propagated virus was infectious for ducks and pigs. This work suggests that some avian species may have had a role in the emergence of PRRS. Unfortunately this work has not been replicated with birds that are common to swine farms. Since PRRSV can be shed in pig feces to some degree, birds may play a mechanical role in the transmission of PRRSV, much like the role that birds are felt to play in the transmission of transmissible gastroenteritis virus. Environmental stability of PRRSV has been reported; it is heat labile with a half-life of less than 30 minutes at 56 C; a few hours at 37 to 39 C; approximately 20 hours at room temperature, and at least several weeks at 4 C (11, 14, 197). PRRSV is relatively stable at -20 C for at least several months and for extended periods of time at -70 C (11, 14, 197). PRRSV is pH sensitive and is most stable at a pH of 6.5 to 7.5 (11, 14, 197). These studies tested PRRSV under ideal conditions for handling the virus, i.e., sterile, buffered solutions in controlled environments. It is assumed that handling virus in less than ideal conditions would be detrimental to virus stability and further reduce its half-life. During an evaluation of potential fomites PRRSV was found to remain infectious for 4 to 6 days in buffer solutions, 9 days in well water and 11 days in city water (132). In contrast, the virus became inactivated within hours of application to porous and non-porous surfaces. These studies suggest PRRSV may be able to survive in wet environments as a potential source of infection, especially during the winter months.

Direct pig-to-pig transmission of PRRSV has been demonstrated in controlled studies in which swine were infected with virus and then at a later date commingled with contact control animals. Detection of virus or virus-specific antibody in the contact control pigs was considered proof that the experimentally infected pigs shed infectious virus during the commingling time span. Under these conditions, transmission of virus has occurred from experimentally infected young pigs (2 to 4 weeks of age) to age-matched contact pigs at 2 (174), 4 (2), 6 (198), and 8 weeks (114, 174) post infection (PE). Contact transmission from experimentally infected gilts to finisher pigs was reported at 99 days PE (205). In piglets born congenitally infected, contact transmission was reported at 9, 12, 14, and 16 weeks post-parturition to age-matched contacts (10). One report described an association between administration of corticosteroids and the transmission of virus approximately 15 weeks post-infection of pigs (2). In this case 4 pigs (which had been infected with PRRSV for approximately 6 weeks) were housed adjacent to a pen of 5 control pigs with fence-line contact from 6 to 15 weeks PE. At this time, 15 weeks PE, the control pigs were commingled with infected pigs; 2 infected and 2 control pigs in one pen and 2 infected and 3 control pigs in the adjacent pen. In the pen of 4 pigs the 2 infected pigs received corticosteroids for 5 days. The control pigs in direct contact with the corticosteroid treated pigs developed PRRSV-specific antibody 2 and 5 weeks later.

At 7 weeks post corticosteroid treatment (conclusion of the study) 2 of the 3 pigs in the adjacent pen were viremic but not seropositive. This study implied that corticosteroid treatment played a role in the transmission of virus; however, in two separate studies the administration of corticosteroids to experimentally infected pigs at 84 and 110 days PE did not result in demonstrable shedding of virus to contact pigs or detectable replication of virus in serum, respectively (173, 174). In all of these contact studies the route of transmission is unknown, however, it is assumed that virus is shed from infected pigs in one or more excretions and susceptible pigs become infected by contact with them or by contact with blood through fighting.

These experimental transmission studies have supported PRRS field investigations that usually concluded the movement of recently infected swine was the predominant mode of virus transmission. Nevertheless, there were many field cases that did not have direct evidence of animal movement as the source of infection; however, most of these cases did have one common risk factor, the purchase of semen from a boar stud that had been recently infected with PRRSV. These observations produced the hypothesis that boars could transmit PRRSV via contaminated semen (48, 147, 196); which if true, could have resulted in rapid dissemination of virus.

Semen is difficult to test for virus by conventional methods because it is very cytotoxic for cell cultures. Of course it can be diluted enough so that it is not toxic, however, if virus is present in semen at a low titer, it could be missed due to dilution effects. One solution to this problem was circumventing cell culture by use of a swine bioassay method involving the inoculation of pigs with aliquots of semen collected from infected boars and monitoring the pigs for evidence of PRRSV-infection (seroconversion) indicating the semen contained virus. With this method shedding of PRRSV in semen has been demonstrated for up to 42 days post infection of the boar (169). PCR assays were developed to circumvent the logistical difficulties of the bioassay model and the toxic effects of virus isolation. This technique was shown to correlate with the bioassay method and it may be more sensitive based on the detection of PRRSV nucleic acid, and presumably infectious virus, in semen up to 92 days post-infection (27). Collectively, it appears that essentially all experimentally infected naive boars have shed virus in semen within a few days of infection for a duration of at least 1 to 2 weeks and some boars have shed virus intermittently for an extended period of time (27). Demonstrating the presence of PRRSV in semen was only half of the equation for confirming semen as a potential mode of virus transmission. The balance of this equation was investigating the susceptibility of sows to a venereal route of transmission.

Gilts were susceptible to an intrauterine/vaginal inoculation of extended semen spiked with either 4.7×10^5 (172) or 4×10^6 TCID₅₀ (145) PRRSV. Gilts have been infected with virus following insemination with raw semen collected from boars experimentally infected with PRRSV (58, 196). In the raw semen studies the dose of virus was unknown; however, it was believed to be less virus than what was used in the PRRSV-spiked semen studies previously described. The quantity of virus may be significant because gilts in 2 studies did not become infected following insemination with aliquots of either raw (58) or extended (171) semen collected during acute experimental infection. In the former study, the raw semen may not have contained infectious virus based on negative bioassay results (58). In the latter study, some of the ejaculates tested positive for virus by bioassay, however, not all of the ejaculates were tested for infectious virus before use leading the authors to hypothesize that the virus titer of the semen was below the threshold of infectivity for a venereal route or the use of semen extenders may have inactivated the virus or the boar shed limited amounts of virus and some of the ejaculates did not contain virus (171). Nevertheless, gilts and sows have been susceptible to an intrauterine/vaginal inoculation of PRRSV that corroborates the field observations that acutely infected boars could transmit virus via semen.

Aerosol transmission of PRRSV has been proposed as a mode of transmission over extended distances (up to 20 kilometers) when there appeared to be no evidence for the movement of infected swine or semen (42, 82). Results from studies evaluating aerosol transmission have been equivocal which may be due more to the pathogenesis of the disease and the interaction of PRRSV with other swine pathogens than to an ability or inability to experimentally replicate field conditions that may promote the aerosol transmission of virus. Pigs and gilts have been infected by an inhalation of aerosolized PRRSV supporting the hypothesis that pigs can inhale virus and become infected (47, 175). In another study pigs acutely infected with PRRSV were only able to transmit virus to pigs in adjacent raised decks with a common air flow in 3 out of 5 attempts despite the pens being separated by only 46 to 102 cm (194). In a separate study PRRSV-infected pigs did not transmit virus from one isolation chamber “downwind” to another chamber containing susceptible pigs even though the two chambers were connected by a tube through which air and potentially virus flowed (Dr. Bob Wills, personal communication). However, pseudorabies virus (PRV) was transmitted under similar conditions from one chamber to the other implying that the transmission of PRV may have been due to virus-induced coughing or sneezing that resulted in aerosolization of the virus. This may be an important point for aerosol transmission since uncomplicated PRRSV infections under laboratory conditions do not produce coughing and sneezing (61, 63). In a separate study PRRSV was

transmitted in 1 of 2 trials through a tube from one chamber containing infected pigs and direct-contact pigs to another chamber housing aerosol-contact pigs. In the one trial that failed to demonstrate aerosol transmission, there was only partial direct contact transmission which led the authors to speculate that the propensity for aerosol transmission could be strain related (177). Due to the presumed interaction of PRRSV and other agents in the porcine respiratory disease complex and to the sheer numbers of swine housed in confinement, the opportunity for aerosol transmission within a barn certainly exists and it is probably a frequent occurrence. However, based on the previously described studies, aerosol transmission between farms, at least in the U.S. is probably a very rare event.

Pathogenesis

Swine are susceptible to infection with PRRSV by the following routes; intranasal, oronasal, intravenous, intramuscular, and by aerosolization. Experimental infections of pigs and sows have reproduced the predominant clinical signs that are associated with PRRS epizootics, i.e., reproductive failure and respiratory disease. Most experimental infections have used an oronasal or intranasal (which may result in some oropharyngeal exposure) route of virus inoculation which is assumed to mimic exposure in the field. Whether this is true or not, swine appeared to respond to experimental infection in a similar fashion as to an uncomplicated field infection, i.e., the host developed mild clinical signs, a prolonged viremia, characteristic lesions, and an apparent slow protective immune response. Due to economics, logistics and the pressure to replicate field observations; most studies have been designed to evaluate the clinical effects of a PRRSV infection with the earliest times for examining tissues occurring around 4 to 7 days PE. Fortunately, a few studies have been completed with the objective of examining the pre-clinical infection.

One of the most notable consequences of a PRRSV infection is a prolonged viremia. Virus has been detected by conventional virus isolation methods in serum from pigs as soon as 12 hours PE (151). In a separate study, virus was isolated from the buffy coat 1 day PE, the earliest time point a buffy coat has been tested (149). In general, virus can be isolated for a longer period of time PE from younger pigs when compared to older pigs and adult swine. The duration of viremia in young pigs infected with PRRSV at 2 to 3 weeks of age is usually 3 to 5 weeks PE (47, 61, 62, 70, 149, 151). Congenitally infected pigs may have a longer duration of viremia, i.e., up to 8-weeks-post partum (107) that does not take into account the length of viral replication *in utero*. The PCR technique may be more sensitive than virus isolation for detection of virus in serum, e.g., in one

study virus was isolated from the serum of congenitally infected pigs for about 7-weeks-post partum, however, PRRSV nucleic acid was detected in the buffy coat by PCR for 210-days-post partum (10).

When compared to young pigs, the duration of viremia in adult swine has been short-lived which probably reflects sporadic or infrequent testing as well as a different host response (22, 25, 27, 107, 112). For example, if a group of sows was tested on day 7 PE, maybe 50 to 75 % of the animals would have a detectable viremia versus day 14 PE when only 30 to 50 % would be viremic and detection of viremia on day 21 PE would be rare. However, if the group was tested more frequently and earlier during the infection, then most if not all of the animals would have a detectable viremia at one time or another.

A second, notable characteristic of PRRSV infections is the expected tropism of the virus for cells of the phagocytic lineage since other members of the Arteriviridae family share this same trait (134). It is assumed PRRSV enters the host through a specific virus/host-cell interaction at a mucosal surface involving some cell(s) of the phagocytic lineage, however, this has not been demonstrated. Following experimental infection, the first identification of PRRSV within a pig's body has been in serum collected 12 hours PE (151) and the first lesions associated with PRRSV have been observed within 24 hours PE (136). Completely assembled virus particles have been observed in alveolar macrophages 9 hours after *in vitro* infection with PRRSV (138). If PRRSV replicates similarly *in vivo*, then the detection of viremia within 12 hours of inoculation reflects a primary and at best, a secondary round of virus replication. The tissue in which this initial replication occurred is unknown although it is probably located either in the hemolymphatic system or intimately associated with it. Obviously, almost all tissues in the pig's body are in communication with the hemolymphatic system, however, the magnitude of the viremia, 10 to 100 TCID₅₀ virus/ml within 12 hours of exposure (151) and the subsequent, rapid development of a substantial virus load within the blood and body (47, 61, 149, 151) implied that the site of virus replication was not focal or sequestered in the pig, but rather diffusely located and intimately associated with the hemolymphatic system.

Based on the apparent tissue tropism of PRRSV and the quick onset of viremia, it is no surprise that within several days of infection virus, viral antigen and nucleic acid would be identified in many tissues throughout a pig's body. In pigs following intranasal exposure, virus was first isolated in serum and lung tissue by 12 hours PE (151), and it has been isolated from serum, buffy coat, lungs, tonsil, spleen, heart, thymus, brain, nasal turbinates, small intestine, urine, feces, nasal and throat swabs, and a variety of lymph nodes by 24 to 96 hours PE (47, 62, 108, 151, 166). Virus has been consistently isolated from serum, lung, tonsil, and a variety of lymph nodes for 2 to 4 weeks

following infection (47, 62, 70, 108, 149, 151, 166) and less frequently from spleen, heart, thymus, nasal turbinates, small intestine, brain, and nasal and throat swabs (47, 62, 108, 149, 151, 166). Virus has been isolated inconsistently from urine and feces during this time span, and there is a single report of virus isolated from bone marrow at 3 days PE (108). During the convalescent phase virus has been recovered from lung tissues for 4 to 6 weeks (47, 108, 155), tonsil for 4 to 6 weeks (61), and serum 3 to 5 weeks (47, 61, 149, 151) following experimental infection. In one study virus has been intermittently recovered from oropharyngeal scrapings for up to 157 days PE (195).

In general, only a few gross lesions and mild clinical signs have been observed in experimentally infected pigs. A mild pneumonia has been reported within 24 hours of infection (136), however, most gross lung lesions were usually first recognized 72 to 96 hours PE as a failure of the lungs to collapse upon opening the thorax, and as a diffuse consolidation of the lung producing a mottled, tan coloration with cranioventral distribution (63, 151). The severity of pneumonia has been variable with gross lesions most pronounced by 10 to 14 days PE and by 21 to 28 days PE they have mostly resolved (61, 63, 149, 151). A generalized lymphadenopathy developed which could be most pronounced in the cervical, tracheobronchial, mesenteric, middle iliac and inguinal lymph nodes. It usually appeared 5 to 7 days PE and was most pronounced from 14 to 28 days PE (61, 63, 149, 151). Anorexia and hyperthermia have been consistent clinical signs following experimental infection in pigs beginning 2 to 4 days PE followed by lethargy and dyspnea beginning 5 to 7 days PE (30, 61, 63, 70, 149, 151). Mild or no clinical signs have been observed following experimental infection (108).

The systemic effects of PRRSV can be appreciated by the observation of microscopic lesions in diverse tissues throughout the pig. In general, the lesions have some commonalties, i.e., an inflammatory nature that is focal (except for the lung) consisting of lymphohistiocytic infiltrates usually associated with the vasculature. Based on gross and microscopic lesions, the lung is the primary organ that is affected by PRRSV resulting in an interstitial pneumonia that can be severe. The second most affected organ system is the lymphatic system resulting in a generalized lymphadenopathy. A number of microscopic studies have been completed characterizing the host response to PRRSV. Lesions have been observed in nasal turbinates by 12 hours PE (151) and in lung by 1 to 2 days PE (61, 136). However, most studies did not examine tissues until 3 to 7 days PE at which time lesions were consistently observed in lung, tonsil, spleen, a variety of lymph nodes and inconsistently observed in liver, stomach, small intestine, brain, and heart (30, 61, 63, 70, 96, 151). Typically the severity of lesions peaked around 2 weeks PE and most were resolved by 3 to 4 weeks

PE, the time when most of the pathogenesis studies in pigs have been terminated. At 4 weeks PE lesions in lung, lymph nodes, and tonsil were still observed (61, 63, 151). Gross and microscopic lesions have been found in lymph nodes of congenitally infected pigs through 40 days post-partum (153). Resolving microscopic lung lesions and active renal lesions have been reported in pigs 7 weeks PE (33). The renal lesions were compatible with lesions that have been described for viral diseases that have immune-mediated pathogenic mechanisms.

Immunohistochemistry (IHC) and *in situ* hybridization (ISH) techniques have been developed for the detection of PRRSV antigen and nucleic acid in swine tissues. In general, the temporal distribution of antigen and nucleic acid correlates with the presence of microscopic lesions and the isolation of PRRSV from tissues. PRRSV antigen has been observed in lung, heart, tonsil, brain, and nasal turbinates at 12 hours PE (150) and by 1 to 3 days PE through 3 to 4 weeks PE, antigen and nucleic acid have been consistently identified in lung, tonsil, and lymph nodes; less consistently in spleen, heart, small intestine, kidney, liver, thymus, brain, aorta, nasal turbinates, and stomach; and infrequently reported in the adrenal gland (47, 59, 62, 65, 93, 96, 136, 150, 166). PRRSV nucleic acid was identified in lung and tonsil tissue up to 6 weeks PE (the conclusion of the study) and PRRSV-specific antigen was observed through 4 weeks PE (166). The authors of this study suggested viral nucleic acid may persist in select tissues for a longer time than viral antigen and infectious virus. The potential mechanism and significance for this proposed persistence of PRRSV-specific nucleic acid is unknown.

Antigen and nucleic acid were observed in individual cells and in small foci of cells that may or may not have been associated with lesions. Generally, when the morphology of infected cells could be determined or the type of infected cell was identified by specific monoclonal antibodies, they were determined to be either macrophages or related antigen processing cells located in many organs (47, 59, 62, 65, 93, 96, 136, 150, 166). Other cell types containing PRRSV-specific antigen or nucleic acid have occasionally been observed, e.g., monocyte, myocytes, type II pneumocytes, bronchiolar epithelium, and arteriolar endothelium (59, 62, 136, 150, 166).

Except for obvious differences, boars and sows have responded similarly to PRRSV infection, i.e., they were susceptible to infection, they displayed few clinical signs, and the duration of viremia and the onset of an immune response were similar. Experimentally infected boars have shed virus for up to 43 days (169) and PRRSV nucleic acid for up to 92 days (27) PE in semen indicating that PRRSV was sequestered somewhere in the boar for an extended period of time. Since a detectable viremia has been relatively short-lived in the boar, the presence of PRRSV in semen was believed

due to either a sub-detectable viremia and emigration of virus or virus-laden cells from the circulatory system into the urogenital system, or virus was excreted from sites within the reproductive tract. Limited studies have supported the latter hypothesis; i.e., virus replicated in tissues within the reproductive tract and was excreted with the ejaculate. In one study virus was isolated from the bulbourethral gland from 1 of 4 experimentally infected boars 101 days PE; interestingly, this was the same boar that had detectable nucleic acid in its semen through 92 days PE (27). However, in another study 4 boars were necropsied 56 days PE (13 to 43 days after the last detectable shedding of virus in semen) and virus was not isolated from samples of the testes, epididymis, ductus deferens, seminal vesicles, bulbourethral gland, prepuce, penis, bone marrow, spleen, kidney, or lungs (169). Based on ISH, virus replication has been demonstrated in the testes of experimentally infected boars (167). In this study, viral nucleic acid and antigen were detected in apparent macrophages in the interstitial tissues of the testes and in germ cells of the seminiferous tubules; specifically, spermatogonias, spermatids, and spermatocytes. Surprisingly, PRRSV was not associated with mature spermatozoa, but was associated with nonspermatozoa cells (immature sperm progenitor cells, multinucleated giant cells and cytoplasmic droplets). This observation is supported by earlier reports describing PRRSV nucleic acid associated with the cell fraction of semen including spermatozoa (29) and the cell fraction of semen without spermatozoa (157). Other than germ cell death and hypospermatogenesis observed in one study (167), no gross or microscopic lesions have been associated with experimental PRRSV infections in boars, although the effect on semen quality has been variable following infection. Following experimental infection with field virus, effects have ranged from none (169, 171) to a slight decrease in semen volume (196) to a significant decrease in sperm motility and spermatozoa with normal acrosomes and a significant increase in the number of proximal droplets and abnormal tails (141). Infection with MLV has resulted in significant decreases in forward movement and normal spermatozoal morphology (28). Collectively, these boar studies have supported field observations in which boars generally have mild clinical signs following infection and may or may not have a decrease in semen quality (49).

A number of studies have been completed which involved the exposure of pregnant swine to PRRSV. Most of these were designed to test the pathogenicity of a field or vaccine isolate and only a few were designed to investigate the pathogenesis of maternal reproductive failure. Exposure of swine to PRRSV during the last third of gestation (≥ 76 days of gestation) has consistently produced the clinical signs associated with a PRRS epizootic, i.e., anorexia and pyrexia in gilts and sows, late-term fetal death, high incidence of stillborns, premature farrowings (GD 108 to 112), weakborn pigs,

and some abortions (expulsion prior to GD 108) (13, 15, 25, 35, 107, 112, 135, 175, 197). Without question these studies have verified the field observations of late-term reproductive failure, however, the linkage between PRRSV and the abortion storms and infertility associated with PRRS epizootics has not been so forthcoming. This may be simply due to small experimental groups since an abortion storm for a producer involving 5 % of the pregnant animals in a 2,500 sow herd would be dramatic; however, it would be difficult to reproduce this incidence rate in a laboratory with only a few animals in a group.

Although few extensive reproductive pathogenesis studies have been completed, much can be deduced from experiments evaluating the pathogenicity of PRRSV isolates. Some piglets born to dams experimentally infected between GD 85 to 93 contained PRRSV-specific antibody at birth which demonstrated fetuses were immunocompetent to PRRSV during late gestation. Young pigs develop a PRRSV-specific immune response within 2 weeks of infection (61, 63, 108, 149, 151). Assuming fetuses in late gestation develop antibody at a similar rate, then seropositive pigs at birth would have been infected with PRRSV for at least 14 days. In the case of gilts exposed to virus at GD 90 (15, 107, 112), the seropositive pigs at birth were probably infected with virus within 10 days of the dam's exposure to virus. Porcine fetuses are capable of developing an *in utero* protective immune response against some pathogens, e.g., porcine parvovirus fetal infections after GD 70, however, this is probably not the case for PRRSV since some fetuses that died were seropositive.

The potential routes for fetal exposure are either transplacental or intrauterine. Several possibilities exist for the mechanism of transplacental infection: 1) virus could be non-cell associated and simply diffuse across the maternal-fetal junction; 2) virus could propagate across the maternal-fetal junction, i.e., virus could infect a maternal cell which would fuse to an adjacent cell and transfer progeny virus, this cell to cell transfer of virus could cross the barrier; and 3) virus could be associated with a maternal cell that would migrate through maternal tissues and penetrate fetal tissue. All of these scenarios result in the transfer of infectious virus into fetal tissues and a subsequent fetal infection with the possibility of intrauterine spread of virus. Assuming intrauterine spread of virus does occur, it could involve intimate contact between the placental tissues of the infected fetus and the adjacent non-infected fetus; however, since there is no apparent exchange of fluids between fetuses the mechanism for the putative transuterine spread of PRRSV is unknown.

Based on testing presuckle sera, the incidence of *in utero* infected fetuses in a litter is variable. Moreover, many fetuses die *in utero* following exposure of the dam and it is difficult to isolate PRRSV from them because the virus is believed to be very labile in postmortem tissues. In these

cases it is assumed the fetuses died as a result of the dam's exposure to PRRSV resulting in fetal infection and subsequent death. Examination of dead fetuses has revealed a spectrum of postmortem changes ranging from "fresh appearing fetuses" that apparently died within a few hours or a day of expulsion to fetuses that have undergone extensive postmortem autolysis and were dark brown in coloration, edematous, and had begun the mummification process. Based on previous studies involving late-gestation fetal death (87), a rough estimate can be made on how long these PRRSV-infected fetuses may have been dead in utero. In general, the edematous, discolored fetuses have probably been dead for several days which implies in the case of infection of the dam at GD 90 and the delivery of autolytic fetuses at GD 114, that the autolytic fetuses were infected and died within 20 days of exposure of the dam.

The high incidence of stillbirths (intrapartum deaths), which has been one of the predominant clinical signs reported in PRRS epizootics, has also been a significant part of the reproductive failure reported in experimental infections. In experimental infections of the dam not all of the stillborn fetuses were infected (25, 107, 112, 175) suggesting the high incidence of stillbirth may not be solely due to fetal infection, but could be due to virus effects on the dam resulting in a primary inertia or the infected fetuses were unable to actively participate in parturition due to weakness or death, all of which could result in a delayed parturition which may contribute to the high incidence of stillborns.

The delivery of weak-born, infected pigs that usually die within 1 to 2 days of birth has been commonplace in field and experimental infections. Generally, the piglets have been free of gross and microscopic lesions resulting in a questionable cause of death. Frequently by 1 to 2 days of age some of the congenitally infected pigs will develop the thumping that has been almost pathognomonic for PRRS. In these affected pigs a significant pneumonia develops which has been diagnosed as the cause of death. Microscopic lesions have been infrequently observed in aborted or stillborn fetuses collected from PRRS epizootics (152). Simply stated, they have been described as myocarditis, encephalitis, and a vasculitis in lung, heart, and kidney. The apparent low incidence of these lesions might question their diagnostic significance, however, they may be underreported due to poor sampling techniques or the lesions are very labile and can be confounded by autolysis.

The mechanism(s) for the PRRSV-induced abortions have not been elucidated. After experimental infection of gilts and sows during late-gestation, transplacental infection with fetal death has been frequently reproduced, however, only a few abortions have occurred in these studies (25, 175). In one, a mild myometritis was found in sows that did abort (25), otherwise no significant maternal or fetal lesions have been found following experimental infection of the pregnant sow. A

mild endometritis, myometritis, and placentitis have been observed in sows naturally and experimentally infected (164). PRRSV has been isolated from ovarian tissues at 10 and 20 days PE (144) and viral antigen has been observed in ovaries 5 days PE (172). Abortions have been attributed to a generalized maternal illness induced by PRRSV and not to any specific lesion, however, most gilts and sows appear normal at the time of abortion which questions this putative abortion mechanism.

In field cases and experimental studies late-gestation reproductive failure has received the most discussion. Few field reports have specifically described reproductive failure during mid-gestation (GD 39 to 75) that would be recognized as aborted fetuses with a crown-rump length measurement of 6 to 18 cm, or assuming the fetuses became transplacentally infected and subsequently died like fetuses during late gestation, it would be recognized as a high incidence of mummified fetuses that would have a somewhat smaller crown-rump length than would be predicted depending on how much they may have shrunk during the mummification processes. Limited studies have been completed evaluating the effects of PRRSV during the mid-gestation phase. In one study 8 sows were intranasally exposed to PRRSV between GD 45 to 50; 2 sows were necropsied at 1, 2, and 3 weeks PE and none of the fetuses in these litters were transplacentally infected (22). The remaining 2 sows farrowed 65 and 67 days PE and PRRSV was isolated from 2 fetuses in one litter and PRRSV-specific antibody was detected in 2 stillborn pigs in the other litter. A separate study had similar results; gilts were intravenously exposed to PRRSV at GD 30 (3 gilts), 50 (3 gilts), 70 (3 gilts), and 90 (5 gilts) and were either necropsied at 3, 6, and 9 weeks post-exposure or allowed to farrow (107). Seven of the 14 gilts had transplacental infection; 1 gilt exposed at GD 50 and necropsied 6 weeks PE, 1 gilt exposed at GD 70 and necropsied 6 weeks PE, and all 5 gilts exposed at GD 90. One entire litter from a gilt exposed to virus at GD 30 was mummified at 6 weeks PE, although the cause of the fetal deaths in this litter was not known, porcine parvovirus was ruled out. These studies, in conjunction with the experimental infections of gilts and sows during late-gestation, implied that the incidence of transplacental infection and subsequent fetal death increased with the length of gestation, i.e., more fetuses became infected and affected when the dam was exposed to PRRSV during late-gestation than during mid-gestation.

An explanation for this has been attributed to an apparent “thinning” of the maternal-fetal junction as pregnancy advances (22). There are varied opinions about the nomenclature used to describe the maternal-fetal junction, for the purposes of this discussion it will be referred to as the placental barrier. The porcine placenta is classified at the microscopic level as an epitheliochorial

placenta referring to the 6 anatomic layers between maternal and fetal circulation composing the placental barrier. These layers, in order from maternal to fetal circulation, are endothelium of maternal capillaries, maternal connective tissue, uterine epithelium, fetal chorionic epithelium, fetal connective tissue, and fetal endothelium. This anatomical description includes maternal and fetal tissues referred to as the maternal and fetal placenta (3 layers each) that function as one organ within the sow, the placenta. The placenta has been estimated to be approximately 6 μm thick during early to mid-gestation and as pregnancy advances it may thin to about 2 μm , essentially the maternal capillaries can become opposed to fetal capillaries with only uterine epithelium and fetal chorionic epithelium separating the two distinct vascular systems. This closer association improves the transfer of maternal nutrients, and possible pathogens as well.

As previously discussed, semen abnormalities have been detected in some experimentally infected boars that could explain in part why poor conception rates have been reported during PRRS epizootics. In addition to the proposed paternal cause (poor semen quality) of conception failure, there could be a maternal component that may involve ovulation, fertilization, and implantation of the embryo. Moreover, the maternal role may be significant since late irregular returns to estrus have been reported as a major consequence of PRRS implying the dam was pregnant but did not maintain pregnancy. Irregular returns to estrus would have little to do with the boar unless the litter size was too small because of poor semen quality.

The effects of PRRSV infection on conception and early gestation have been investigated. The hypothesis of semen as an important mode of virus transmission has been supported by gilts becoming infected with PRRSV by way of intrauterine exposure with PRRSV-spiked semen, or semen collected from acutely infected boars. One report described 2 gilts that were infected following insemination with raw semen collected from an acutely infected boar, both became clinically affected and did not conceive which implied PRRSV may have had some effect on conception, however with only 2 gilts and no controls, no conclusions could be made (196). A preliminary study evaluating the acute affects of PRRSV on conception, infected gilts at the time of insemination with either virus-spiked semen ($n=5$) or with semen collected from a boar 1 week post-infection with PRRSV (172). Five days PE the gilts were necropsied and 2 and 4 of the gilts in their respective groups were pregnant. An additional study that used PRRSV-spiked semen to infect gilts also concluded PRRSV had no effect on conception rates (145). Moreover, intranasal and intravenous exposure to PRRSV at the time of insemination had no effect on conception rates (144). Further studies tested the direct effects of PRRSV on 4 to 16 cell stage porcine embryos and found

no detrimental effect when the embryos were bathed in virus or microinjected with virus (142). This study concluded that early embryos were not susceptible to PRRSV due to the failure of virus to penetrate the zona pellucida and the apparent lack of cells permissive for PRRSV growth in the 4 to 16 cell stage embryo. These results could explain the lack of PRRSV-induced conception failures reported in the experimental studies. However, once embryos hatch from the zona pellucida and begin implantation, they may become susceptible to PRRSV. Gilts exposed to PRRSV at the time of insemination and necropsied 20 days later had some embryos infected with PRRSV, although gilts treated similarly and necropsied 10 days PE (prior to hatching) did not have embryonic infection (144). Although the incidence of fetal infection was low it was supported by a second study where gilts were artificially inseminated and exposed to PRRSV at 1, 2, or 3 weeks of gestation and necropsied 3 weeks PE (140). Again, transplacental infection was found at a low incidence. Collectively, these studies investigating the effects of PRRSV on conception and early gestation (0 to 38 days of gestation) indicated that PRRSV may have little direct maternal effect on conception and most of the decreased conception rates in the field associated with PRRS epizootics may be attributed to poor quality semen. Results from these early gestation studies suggested the fetus was susceptible to transplacental infection essentially from the beginning of placentation, although at a much lower incidence rate during early gestation when compared to late gestation. Based on these studies it is possible that the increased incidence of late irregular returns to estrus reported during PRRS epizootics was related to maternal PRRSV infection with an occasional loss of early gestation pregnancy due to dying fetuses or potentially to abortions.

Diagnosis

Initially, the etiology of PRRS was unknown and it was diagnosed based on the presence of clinical signs during an epizootic (late-gestation reproductive failure and pneumonia in pigs) and poor production performance during an enzootic PRRS infection. Although not pathognomonic, interstitial pneumonia observed in pigs was very suggestive of a PRRSV infection resulting in a valuable diagnostic lesion that has remained important even after the development of more definitive methods to diagnose PRRS. The ability to culture virus and produce viral antigens led to the creation of several serologic methods for detection of PRRSV-specific antibody(1, 11, 77, 130, 189, 199, 200). Assays utilizing IHC, PCR, and ISH have been developed which can identify viral nucleic acid or antigen in tissue samples that are formalin fixed (64). With these tools plus the ability to isolate

virus, the presence of virus within a herd could be identified and the next obstacle was determining the correct selection of diagnostic samples.

The knowledge gained from experimental infections has been useful for determining sample selection. Essentially all swine experimentally infected with PRRSV have seroconverted within 2 weeks of challenge. This immune response is believed to mimic field infection and has diagnostic significance in a seronegative herd since paired serum samples collected at least 2 weeks apart would demonstrate seroconversion in infected animals. The use of serology in a seropositive herd becomes problematic because of the normal PRRSV immune response in swine and the use of modified-live vaccines (MLV). Limited field studies indicated swine have a maximum rise in antibody titer approximately 4 to 6 weeks PE to field or vaccine virus followed by a titer decay that can drop below detectable levels within 4 to 6 months (2, 41, 163). Because of decreasing antibody titers, it is best to bleed animals as soon as possible after clinical signs have been noticed. Based on experimental studies as previously described; anorexia, pyrexia, and dyspnea can occur within the first week of infection and abortions have occurred around 2 to 3 weeks PE. Therefore, testing animals during the onset of clinical signs and again 2 to 3 weeks later, should detect rising antibody titers, a good indication of recent infection.

In general, virus can be isolated from serum of pigs for 2 to 4 weeks after infection, therefore serum is a good sample to test for virus from clinically affected pigs during an epizootic of PRRS. However, adult swine have a much shorter, detectable viremia resulting in a poor isolation rate from serum that was collected at the time they were clinically affected. Lung lavages provide a good specimen to test for virus. Under experimental conditions lung lavages are a more sensitive specimen than serum to detect virus replication either by testing the lavage sample for virus or by cocultivating the lung lavages with a permissive continuous cell line (109, 112, 113). The use of alveolar macrophages in conjunction with a continuous cell line may enhance success since some field isolates exist that grow preferentially in either the cell line or alveolar macrophages (7).

The advent of MLV has complicated diagnostics since vaccine virus can replicate like field virus in pigs resulting in a need for differential diagnostic tests. Currently, there are two methods that attempt to differentiate virus strains. One involves comparison of strains at the nucleotide sequence level and the other compares the restriction fragment length polymorphism (RFLP) of the strains (190). The RFLP test has been utilized for differentiating one of two commercially available MLV from field strains of PRRSV (190) and a monoclonal antibody test has been reported to differentiate the other MLV from field strains (128).

Probably the leading cause of diagnostic failure for PRRS has not been a technology failure, but rather, a failure to collect and submit an adequate sample. Under experimental conditions there have been dramatic variations among individual animals in their susceptibility to virus infection and clinical response. Under field conditions these variations are probably accentuated even more and when coupled with unknown exposure times, the probability of collecting the best diagnostic samples, i.e. samples collected during maximum virus replication, would be very low.

Prophylaxis

Currently in the United States there are several options available to swine producers for immunizing their herd against PRRS; there are 2 MLV and 1 killed virus (KV) vaccine commercially available and there are several veterinary biologic companies that produce killed autogenous virus vaccines for individual farm use. In addition to vaccines, there is a rapidly growing body of work concerning the prevention of PRRS through management practices (curtailing the movement of animals and the development of isolation and acclimatization protocols for incoming animals) that may or may not involve the use of vaccine. Generally, these management practices are tailored for individual farms resulting in many permutations that all have a central theme, i.e., trying to prevent the introduction of PRRSV into the herd by controlling the inflow of infected animals and trying to stimulate protective PRRSV immunity in naive animals by vaccination or natural exposure to virus through contact with cull sows, young pigs, and “feedback” strategies. The balance of this section will be devoted to what is known about the PRRSV-specific immune response of swine and how that information could be related to management practices.

As previously stated, essentially all swine develop an immune response to PRRSV following experimental infection or vaccination with MLV, at present there is limited published information characterizing the immune response to KV. Although based mostly on anecdotal information, the acute humoral immune response for field infections is believed to mimic experimental infection inducing class IgM and IgG antibodies within 5 to 7 and 10 to 14 days of infection, respectively (76, 130). Although the acute humoral immune response appears similar following any live PRRSV exposure, there may be a difference in the convalescent antibody response between an experimental infection and a field or MLV infection. The antibody response following experimental infection with field virus has remained positive throughout the duration of all reported studies, i.e., antibody was detected at all time points, in some cases out to about 1 year (127). In contrast, field reports indicate the antibody titer following vaccination or field exposure in some swine decayed to sub-

detectable levels within 4 to 6 months (2, 41, 163). The cause for this apparent difference is unknown although it could involve the pathogenicity of the strain and possibly the dose of virus administered. However, it is difficult to imagine how the dose would have much effect since swine respond to vaccine or field infection with a viremia that could last for several weeks followed by extended periods of time when virus could be actively replicating in the lung, tonsil, lymph nodes and possibly other tissues. This persistent infection should result in a sustained source of antigen for the immune system that would surpass the initial challenge virus dose.

Although the PRRSV humoral immune response can be clearly measured, the protective significance of these antibodies is poorly understood. Virus neutralizing (VN) antibodies are generally thought of as protective for the host, i.e., the development of VN antibodies would coincide with clearance of virus from the host. In the case of PRRS, VN antibodies usually develop within several weeks of infection, however, they may coexist with circulating virus for several additional weeks before serum virus titers decline below detectable levels (77, 199, 201). These observations have questioned the protective properties of PRRSV-specific VN antibodies. In fact, PRRSV-specific antibodies have been implicated in the pathogenesis of this disease through a mechanism called antibody dependent enhancement (ADE), a pathogenic mechanism that has been described for some viral diseases. Briefly, ADE can be defined as follows; antibody-antigen (virus) complexes bind to Fc receptors on macrophages through the immunoglobulin molecule. This complex is then internalized into the macrophage thus infecting the macrophage with virus that did not enter the cell through a virus-host cell mediated pathway. The phenomenon of ADE may help explain the occurrence of clinical PRRS in herds that have been vaccinated and presumed protected from clinical disease. This hypothesis has been supported by several studies.

ADE has been demonstrated *in vitro* by comparing virus yield from alveolar macrophages that have been exposed to either PRRSV or PRRSV-antibody complexes. In these studies the addition of antibody produced higher virus yields supporting the role of ADE (203). One *in vivo* study involved the inoculation of fetuses at GD 45 to 50 by either intramuscular or intraamniotic injection with either virus or a virus-antibody mixture (22). Significantly more virus was detected in fetuses infected with the virus-antibody mixture when compared to fetuses that received PRRSV with no antibody treatment. This result lead to a conclusion that ADE was responsible for this difference. However, the authors did not speculate on what fetal cell(s) may have possessed Fc receptors at the time of inoculation that would have enhanced virus replication. A second *in vivo* study supporting ADE involved the injection of known amounts of PRRSV-specific antibody into pigs producing 2

groups of pigs with either virus-neutralizing or subneutralizing humoral antibody levels. Based on the magnitude of viremia following virus challenge, pigs that had a subneutralizing humoral antibody titer experienced ADE when compared to pigs that did not have antibody (controls) or had virus-neutralizing levels of antibody (202). In this study, no clinical signs were observed in any group of infected pigs. Preliminary studies have suggested that antibodies directed against the GP5 protein may be mediators of ADE (202) and as one might expect, the heterogeneity of PRRSV may produce variation in the susceptibility of field isolates to ADE (203). Although it may be difficult to evaluate the potentiating clinical effects of ADE, it is clear that PRRSV-specific antibody is not necessary for PRRSV to cause disease as evidenced by many studies which have involved naive animals.

The cellular immune response to PRRSV is poorly understood. Limited studies have been completed that described a specific T cell response to PRRSV (9). A primary T cell response was detected beginning about 4 weeks PE that lasted for about 7 weeks. A secondary T cell response was detected in response to homologous antigen administered 20 weeks post primary exposure, it was detectable for about 3 weeks. This secondary specific T cell response is in contrast to the lack of a secondary humoral immune response to administration of homologous antigen that was reported in this study (9) and by others. The rise of the primary T cell response coincided with the development of VN antibodies which clouded the role of each immune response in the apparent clearance of circulating virus from the blood. Based on preliminary studies, the CD4+ and CD8+ T cells played a major and minor role, respectively, in the specific cellular immune response to PRRSV. A delayed-type hypersensitivity response has been demonstrated in PRRSV-infected pigs indicating an *in vivo* cellular immune response (9).

In addition to the reproductive and respiratory disease, field reports have described a third, loosely defined condition that has been attributed to PRRSV which is immunosuppression. Clinically the immunosuppression is characterized as a resurgence of endemic diseases within the herd resulting in significant losses. Unlike PRRSV-induced reproductive and respiratory disease, the immunosuppression has been difficult to produce experimentally. An early study reported that PRRSV infection in pigs enhanced antigen-specific responses (120). This work was followed by a study utilizing a *Streptococcus suis* pig infection model. The dual infection of PRRSV followed 5 days later with *S. suis* resulted in more affected pigs than with either agent by itself. The synergism between *S. suis* and PRRSV has been interpreted as immunosuppression (51). This experimental design has been repeated with PRRSV administered to young pigs followed by a challenge 5 to 7

days PE with a number of pathogenic organisms; e.g., *Haemophilus parasuis* (32, 158), *Actinobacillus pleuropneumoniae* (137), *Pasturella multocida* (19, 32), *Salmonella choleraesuis* (32), *S. suis* (32), and *Mycoplasma hyopneumoniae* (179). In these studies no synergistic interaction was detected suggesting PRRSV did not significantly predispose pigs to a more severe disease. However, in 2 additional studies a synergistic interaction was reported with dual infections involving PRRSV and *S. choleraesuis* (193) or *M. hyopneumoniae* (176). One difference in these two studies was the administration of the pathogenic organism prior to PRRSV exposure which may account for the contrasting results with the previously mentioned dual infection studies. However, it is possible that biologic variation (challenge strains, pigs, methodology, etc.) or pure chance may account for the divergent results. Divergent results have been reported for dual infection studies involving swine influenza virus and PRRSV. In one study 1 of 3 trials produced a significant interaction (137) and in a second study all 3 trials produced significant interaction although there was considerable variation in clinical signs among the 3 trials (181). Additionally, in the second study a significant interaction between PRRSV and porcine respiratory coronavirus was found (181).

At present, results have been equivocal for pig studies demonstrating PRRSV-induced immunosuppression; this has led to the design of a different challenge model utilizing 5-day-old naturally-farrowed, colostrum-deprived pigs congenitally infected with PRRSV. With this method a synergism between PRRSV and *S. suis* was demonstrated (101). It remains to be seen if this model will be more successful than the conventionally raised pig challenge models for demonstrating apparent immunosuppression.

Although the PRRSV immune response is only partially understood, it can be measured indirectly by evaluating the protective immune response in swine. Swine have been successfully immunized against a PRRSV infection, thus verifying field observations reporting convalescent animals were protected against subsequent exposure to virus (163). Essentially all of these protective immunity studies have involved vaccine trials in which animals were vaccinated with MLV and exposed to challenge virus at a later date. Perhaps the most clear-cut examples of protective immunity have been the vaccination of gilts with MLV followed by field virus challenge. Vaccination of gilts twice prior to breeding with MLV protected them and their litters from a homologous virus challenge (vaccine parent strain) at about GD 90 (54, 66), the most susceptible stage of gestation for PRRSV-induced reproductive losses (107). In these homologous challenge studies the vaccinated gilts farrowed normal litters apparently free of challenge virus. In comparison, the non-vaccinated challenge gilts suffered significant reproductive losses. Heterologous vaccine challenge studies have

been completed with less clear-cut results. Again, gilts were vaccinated twice prior to breeding with MLV and then challenged with either homologous or heterologous virus (field virus that could be differentiated from the homologous virus strain) (54, 68). The vaccinated gilts challenged at GD 90 with homologous virus had normal litters, however the vaccinated gilts challenged with heterologous virus had some litters transplacentally infected with subsequent reproductive losses, although these losses were not as severe as the losses reported for the non-vaccinated homologous and heterologous virus-challenged gilts. These results suggested that vaccination prior to breeding could develop some heterologous protection, but it was incomplete when compared to homologous challenge/protection. An early report about the safety of a MLV described the vaccination of gilts at about GD 90 followed by the delivery of normal pigs that could not be clinically differentiated from control pigs farrowed from nonvaccinated gilts (54). This safety study led to several studies that were designed to test the extensive field practice of vaccinating the dam during the first half of gestation for the prevention of reproductive losses during the second half of gestation.

One experiment replicated the safety study by oronasally exposing gilts to MLV at GD 90 and evaluating their pigs at farrowing (112). In this experiment the pigs appeared normal following birth as reported previously, however, the piglets were tested for virus at birth and some of them were congenitally infected which suggested the MLV was not as safe for use during late gestation as once thought. A follow up study vaccinated 4 gilts at GD 60, a stage of gestation when fetuses were considered less sensitive to transplacental infection, again the piglets were normal at birth and no congenital infection was detected in the 4 litters, however, one pig out of the 4 litters was infected postpartum either by contact with the gilt, possibly via milk, or the pig had a congenital infection that was not detected (110). In this vaccination study, an additional 4 gilts vaccinated at GD 60 were challenged with heterologous virus at GD 90, 30 days after they were vaccinated. Two of the 4 litters each contained 1 known congenitally infected pig, but by weaning, all litters contained mostly infected pigs suggesting that there was some intra-litter spread of virus and/or possible transfer of virus from dam to pigs despite the ingestion of colostral antibody. In a separate, but similar study, gilts were vaccinated at about GD 50 and challenged with heterologous virus at about GD 90 with the same virus strain used in the previously described GD 60 vaccine study. The results were similar in that the gilt vaccination did attenuate the effect of late-gestation challenge, but it did not completely protect the fetuses or prevent the intra-litter spread of virus (54).

Collectively, these vaccine studies demonstrated MLV can induce homologous protection and limited heterologous protection against field virus challenge during late gestation. The limited

heterologous protection may have a temporal relationship, i.e., gilts vaccinated and challenged 30 days later had less heterologous protection than gilts vaccinated prior to breeding and challenged about 120 to 140 days later.

A similar MLV response has been reported for boars. Boars vaccinated with MLV shed vaccine virus in semen although the vaccine virus infection appeared to be limited in duration and magnitude when compared to field virus infection (28, 74, 129, 157). Moreover, the MLV attenuated or prevented the detectable replication of and seminal shedding of homologous challenge virus in the vaccinated boars, a protective homologous immune response analogous to vaccinated gilts (28, 74, 129, 157).

A number of pig MLV vaccine trials have been reported that described the efficacy of vaccinating young pigs once followed by field virus challenge usually 2-4 weeks later (53, 55, 67). The parameters evaluated in these studies usually involved weight gains, pyrexia, clinical evaluations, measurement of specific white blood cell populations, gross lung lesions, viremia, etc. Based on these parameters, vaccinated pigs have been protected against homologous as well as heterologous field virus challenge. The apparent difference in the time necessary for the pig to mount a protective immune response (< 30 days) versus the time necessary for the gilt (> 30 days) has not been reconciled although it could be due to the fact that pregnant animals may be a very sensitive model for detecting protective immunity when compared to the challenge models for young healthy pigs since only 1 infectious virus particle may be capable of causing transplacental infection and thus, clinical signs in the gilts.

As with any MLV there exists the possibility of the vaccine virus reverting to virulence. In the case of PRRS this scenario was rumored to have occurred on a number of occasions following the distribution of the first MLV in North America. However, there are only two reports of vaccine virus transmitting from pig-to-pig in a controlled environment (112, 178). Although the vaccine virus was transmitted among animals and pens, no clinical effects were reported. This is in contrast to a report about MLV transmission among Danish swine in which clinical signs were attributed to infection with vaccine virus (16). The clinical signs were mostly reproductive failure, i.e. dead fetuses, stillborns pigs, and weakborn pigs at the time of farrowing. In these cases, vaccine virus was probably transmitted to pregnant gilts and sows from previously vaccinated young pigs in the same herd and/or from the use of semen collected from vaccinated boars.

Planned exposure to field virus circulating within an endemically infected herd has been promoted as a method to immunize swine against PRRS. Based on the previous discussion about

homologous protection with MLV this concept, immunizing swine with the endemic virus, should be efficacious; however, the safety of this practice has been a major issue as well as the crude exposure methods (feedback of feces, dead fetuses, and fetal membranes from affected sows). Moreover, the acclimatization of incoming animals to cull sows or pigs may not be efficacious for exposing and infecting animals. To date, no studies have been reported that have evaluated these practices.

Killed virus vaccines have been developed in response to safety concerns about MLV and the safety and efficacy of planned field-virus exposure. Limited information is available about 2 commercially prepared KV, one produced in Spain and the other in the United States. In one controlled study the Spanish KV was administered to gilts during early gestation followed by a field-virus challenge between GD 67 and 85. When compared to non-vaccinated challenged controls, the vaccinated gilts had a significant attenuation of reproductive losses. However, the report is not clear about which animals were challenged at what stage of gestation. This may have some bearing on the results since previous pathogenesis studies have indicated the later in gestation when pregnant swine are challenged, the more susceptible their litter is to transplacental infection. Therefore, some of the gilts in the KV study may have been challenged with virus at a time when the incidence of transplacental infection would be less, thus affecting the results. In a second Spanish study KV administered twice to gilts before insemination with PRRSV-spiked semen did not protect them from infection with challenge virus or block infection of some of their embryos (145). Two reports exist about the United States commercially available KV. One involved 4 vaccinated boars which suggested vaccination with KV may inhibit the replication and shedding of challenge virus in the vaccinated boars (170). The other study involved gilts that were vaccinated twice during mid-gestation and challenged with field virus at or about GD 85 (21). A significant reduction in reproductive failure was reported in the vaccinated group when compared to the non-vaccinated challenge controls.

Recently, the use of autogenous KV has become a popular choice for swine producers. The basis for this practice has been the demonstration of homologous protection in controlled MLV studies combined with the safety of an inactivated vaccine and the perceived lack of MLV efficacy in some field cases. The efficacy of these autogenous KV products is unknown, at best there is limited anecdotal evidence to support their use.

Although most successful PRRS control programs have involved the use of MLV, essentially all of them have involved a change in animal flow for the express purpose of reducing the risk of virus entry. This common link of controlling animal movement suggests the careful screening and

isolation of incoming animals may be one of the most important actions implemented by a producer in their fight against PRRS.

CHAPTER 3. PATHOGENESIS OF *IN UTERO* INFECTION IN PORCINE FETUSES WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

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Abstract

Porcine fetuses were exposed *in utero* to porcine reproductive and respiratory syndrome virus (PRRSV) at stages of gestation ranging from 34 to 85 days and examined 17 to 31 days later to determine the effect of gestational age on fetal susceptibility. For each of the eight litters tested during the study, all of the fetuses of one horn of the uterus were exposed to virus by intraamniotic injection; those of the other horn were exposed similarly to a sham inoculum that consisted of sterile cell culture medium. Viral infectivity titers associated with fetal tissues collected at necropsy indicated that, regardless of gestational age, the virus had replicated in fetuses exposed intraamniotically. In addition, virus had also spread and replicated in sham-inoculated littermates in three litters. On the basis of these findings it appears that there may be little or no temporal difference in fetal susceptibility to infection with PRRSV. If so, the lack of early fetal death as a commonly recognized feature of naturally occurring cases of PRRS may be due to a greater resistance of early gestational fetuses to the lethal effects of PRRSV, as suggested by this study, and/or a greater likelihood of transplacental infection during late gestation.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a new disease of swine. The predominant clinical signs of a PRRS epizootic include a reproductive component characterized by an increase of late-term abortions and early farrowings with mummified fetuses, stillborn and weak piglets, late returns to estrus, and repeat breeders; the respiratory component has been described as an acute onset of a "flu-like" respiratory infection with a high morbidity for all ages of swine (57, 80). The etiologic agent has been identified as a virus, known as the PRRS virus (PRRSV). It is classified as a member of the proposed virus family, Arteriviridae, whose members include equine arteritis virus, lactate dehydrogenase elevating virus of mice, and simian hemorrhagic fever virus, all

of which share similar morphology, genomic organization, and tissue tropism with the PRRSV (31, 117).

Under experimental conditions, exposure of pregnant swine to PRRSV during the last trimester of gestation has typically resulted in transplacental infection followed by reproductive failure (25, 175). These events appear to be much less common, however, when females are exposed to PRRSV earlier in gestation (22, 107). As a result, it has been suggested that the consequences of infection of pregnant females with PRRSV may reflect a relative placental permeability to the virus during early versus late gestation, or an age difference in the ability of the fetus to support virus replication following transplacental exposure. In the study reported here we investigated the latter by exposing fetuses of different gestational ages to PRRSV by direct intraamniotic injection and then measuring the extent of virus replication at various times post exposure.

Materials and Methods

Virus -- The PRRSV isolate utilized in this study has been previously described as the swine infertility and respiratory syndrome virus (SIRSV) or ATCC-VR 2332 (11). It was propagated in CL2621 cells (Proprietary cell line CL2621, Boehringer Ingelheim Animal Health Inc., St. Joseph, Missouri) maintained with minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 50 µg/ml of gentamicin. Viral inoculum was prepared by adsorption of virus to confluent monolayers of CL2621 cells for 1 h. Infected monolayers were incubated at 37°C in maintenance medium consisting of serum-free MEM and cell cultures were observed daily for cytopathic effect (CPE) until approximately 75% of cells degenerated. For harvesting, cell monolayers were submitted to two freeze-thaw cycles. Cell culture fluid was clarified at 1000 x g for 30 min and supernatant was filtered through a 0.45 micron pore-size membrane. Viral infectivity was determined in a 50% infective dose method as previously described (146). Infectivity titers were approximately $10^{6.0}$ TCID₅₀/mL. Virus (ninth cell culture passage) was aliquoted and frozen at -80°C.

Animals -- Eight pregnant gilts with known breeding dates were purchased in groups of two or three from two commercial herds deemed free of PRRSV infection based upon herd history and serologic tests (Table I). Fetuses were exposed by intraamniotic injection to either PRRSV (principals) or virus-free cell culture medium (controls) as previously described (111). The animal studies were

conducted in accordance with the guidelines of the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

Experimental design –Each uterus was surgically exposed and fetuses were individually palpated and observed to distinguish live from dead. Principal fetuses were exposed by intraamniotic injection to a high or low dose of PRRSV in an alternating sequence. Each injection site was identified by a suture placed in the serosal surface of the uterus, thus marking the relative position of the fetus.

Group 1 consisted of three gilts (1, 2, and 3) that underwent surgery at 85, 65, or 45 days of gestation (DG), respectively (Table I). All fetuses in one horn of each uterus were exposed to an intraamniotic injection of a 1 ml sham inoculum, i.e., the control fetuses. A 1 ml virus inoculum containing either 10^3 or 10^5 TCID₅₀/mL was injected into the amniotic fluids of the principal fetuses in the contralateral uterine horns. The gilts were euthanized 21 days post-surgery (PS) and their uteri recovered and fetuses examined for the presence of virus.

Group 2 consisted of three gilts (4, 5, and 6) that underwent surgery at 49 DG. The virus inoculum for the three litters was prepared from the serum of fetus P-4, a principal fetus from the litter of gilt 3, in the following manner. A 0.5 ml aliquot of the fetal serum containing about 10^6 TCID₅₀/mL PRRSV was inoculated onto a monolayer of CL2621 and allowed to adsorb for 1 h. Following a media change, the cell culture was observed for CPE and virus was harvested as previously described. The virus inoculum had been passed nine times *in vitro*, once through a porcine fetus and once again through CL2621. Its titer was $10^{4.9}$ TCID₅₀/mL. Principal fetuses were exposed to 1.0 ml of either the undiluted virus suspension or a tenfold dilution of virus. Gilts were euthanized on 17, 19, and 21 days PS and fetuses examined for the presence of virus.

Group 3 consisted of two gilts (7 and 8) that underwent surgery at 34 DG and fetuses were inoculated in a similar fashion as in group 1. The virus inoculum consisted of a tenth passage of PRRSV diluted to concentrations of either 10^3 or 10^5 TCID₅₀/mL. Principal fetuses received a 0.2 ml inoculum of either preparation. Control fetuses received a 0.2 ml sham inoculum of virus-free cell culture medium only. One gilt was euthanized 21 days PS and the second gilt at 31 days PS.

Necropsy procedures –Blood samples were collected from the gilts prior to surgery and at the time of euthanasia which was effected by an intravenous overdose of pentobarbital. Each fetus was examined and crown-rump length (CR) determined for an estimation of fetal age (100). Fetuses

were categorized as alive or dead based on detection of a heart beat or obvious postmortem autolysis or mummification. Samples of lung, liver, heart, kidney, and spleen from each fetus were collected. Fetal fluids were collected from the umbilical cord of live fetuses or the thoracic fluids of dead fetuses.

Virus isolation --Virus isolation was attempted on fetal fluids and tissues. A 0.1 ml aliquot of serum or thoracic fluid was added to 48- to 72-hour-old monolayers of CL2621 cells on glass coverslips in Leighton tubes. The coverslips were fixed approximately 20 h later in 24:1 acetone/ethanol solution and observed for viral antigen by indirect fluorescent antibody (IFA) techniques. The primary serum was harvested from a convalescent pig experimentally infected with ATCC-VR 2332 and the secondary serum was a goat serum containing anti-swine IgG fluorescein isothiocyanate-labeled antibody (Dr. P. S. Paul, College of Veterinary Medicine, Iowa State University, Ames, Iowa). Pooled tissues of each fetus were ground with sterile sea sand in mortar with pestle and serum-free MEM added to produce an approximate 20% (w/v) solution. The suspension was clarified at 1000 x g for 30 min at 4°C. The supernatant was collected and 0.1 ml added to a 48- to 72-hour-old-monolayer of CL2621 cells in a 24-well tissue culture plate. The media were changed 12 to 18 h later and the monolayers observed daily for CPE for eight days. Fetuses were considered to be infected if virus was isolated from either their tissues or fluids or both.

Serology --All serum samples and fetal fluids collected from fetuses older than 60 days were heat inactivated at 56°C for 30 min prior to use. Virus neutralization (VN) tests were conducted as previously described (107). Glass coverslips containing PRRSV-infected monolayers of CL2621 cells were used for testing serum and fetal fluids for IFA antibody as described above.

Statistical analysis --Differences in the incidence of abnormal fetuses and mortality rate between fetuses exposed to virus during the first or second half of gestation were analyzed by Fisher's exact test (160). Differences in the CR between live infected fetuses and live non-infected fetuses were analyzed with *t*-tests (161).

Results

All gilts seroconverted to PRRSV following inoculation of their fetuses (Table I). Antibody was not detected by IFA in the fetal fluids examined. At the time of surgery, no obvious fetal lesions

were noted in any of the litters. All gilts recovered uneventfully from surgery and were normal throughout the study. Fetal position was determined in relationship to the suture placed at the transuterine injection site during surgery. Fetuses were identified as control or principal and by their position in the uterine horn in relationship to the uterine body, e.g., the fetus located next to the body in the control uterine horn would be identified as C-1.

TABLE I. Virus neutralizing (VN) and indirect fluorescent antibody (IFA) tests on sera collected at the time of gilt surgery and euthanasia

Gilt	Challenge	Termination	VN ^a		IFA ^b	
			Pre-surgery	Termination	Pre-surgery	Termination
1	85 ^c	21 ^d	<4	56	<4	128
2	65	21	<4	111	<4	128
3	45	21	<4	28	<4	512
4	49	17	<4	11	<4	512
5	49	19	<4	776	<4	64
6	49	21	<4	388	<4	128
7	34	21	<4	<4	<4	512
8	34	31	<4	<4	<4	256

^a VN titer expressed as the reciprocal of highest serum dilution neutralizing CPE induced by 100 TCID₅₀ of virus (mean titer from 5 tests). ^b Reciprocal of the highest serum dilution given which permitted visualization of fluorescent foci in infected cell-monolayers. ^c Day of gestation when fetuses were exposed to virus by surgical methods. ^d Days post surgery when gilt was euthanized.

Group 1 consisted of gilts 1, 2, and 3 euthanized 21 days PS (Table II). In litter 1, three of the five principal fetuses were alive. Of the two dead fetuses, one had died recently (P-4) and one was undergoing mummification. Virus was recovered from all three live fetuses and from only one of the dead fetuses (P-4). All control fetuses were alive and no virus was detected. In litter 2, all of the principal fetuses had died and were mummified; no virus was detected in the principal fetuses. Four of six control fetuses were alive, virus was recovered from one live fetus (C-3) but not the two dead fetuses. In litter 3, all fetuses were alive, although principal fetuses were edematous and hemorrhagic with friable internal organs. Virus was recovered only from the four principal fetuses.

Group 2 gilts 4, 5, and 6 were euthanized 17, 19, and 21 days PS, respectively. In litter 4, five of seven principal fetuses and five of eight control fetuses were alive. Virus was recovered from all

live principal fetuses but not the dead principal fetuses. Virus was recovered from two live control fetuses, C-1 and C-2, but not from the remaining live or dead control fetuses. In litter 5, five of six principal fetuses were alive. Virus was recovered from the live principal fetuses, but not the dead one. Virus was not detected in the three live fetuses or one small (CR = 7.0 cm), mummified fetus in the control uterine horn. Litter 6 resembled litter 3 in that all principal fetuses were alive even though they were grossly affected and virus was recovered from each one. No virus was detected in the control fetuses which were alive and normal in appearance.

Group 3 consisted of gilts 7 and 8 who were euthanized 21 and 31 days PS, respectively. At the time of surgery, seven principal and five control fetuses were thought to be present in the uterus of gilt 7. At necropsy, eight live fetuses were in the principal uterine horn and two live fetuses (C-1 and C-4) and remnants of what were thought to be fetuses C-2 and C-3 were identified in the control horn. A principal fetus was located in the body of the uterus and was not identified by suture. It was misidentified at the time of surgery as a control, and is now identified as P-1, a sham inoculated fetus in the principal horn. Virus was recovered from principal fetuses P-1 through P-7. Virus was not recovered from fetus P-8 or the control fetuses. At the time of surgery, six principal and eight control fetuses were thought to be present in the uterus of gilt 8. When the gilt was euthanized, four live fetuses, one mummified fetus, and the remnants of a sixth fetus were identified in the principal uterine horn. Virus was detected only in the four live principal fetuses. Seven control fetuses were identified in the control horn, two of which had recently died (C-3 and C-7) and were undergoing mummification. No virus was detected in these fetuses and no physical evidence was found for the eighth control fetus thought to be present at the time of surgery.

Of the 39 fetuses positive for virus, virus was isolated from the serum of all 39; whereas virus was isolated from tissue homogenates of only 35. The serum-virus titers ranged from 10^1 TCID₅₀/mL in a fetus (P-1, litter 7) suspected of becoming recently infected by intrauterine spread of virus to 10^7 TCID₅₀/mL in a fetus (P-4, litter 7) that had been exposed to virus for 21 days. Principal fetuses positive for virus were considered infected by their original intraamniotic inoculation, titers of which ranged from $10^{2.3}$ to $10^{5.0}$ TCID₅₀. No discernable differences in fetal susceptibility or mortality were noted between low and high titered inoculums. A significant difference ($P < .05$) was observed in mortality rates between principal fetuses exposed to virus in the first (litters 3-8) and second (litters 1 and 2) half of gestation; however, no difference ($P = 0.08$) was found in the incidence of abnormal fetuses (hemorrhagic and edematous live fetuses or dead fetuses) between the first and second half of gestation. Although the mean CR of live infected fetuses was less than live

TABLE II. Condition^a of porcine fetuses in gilts (litters) 1-8 at the time of necropsy.

Litter	Infected uterine horn								Control uterine horn							
	P-8	P-7	P-6	P-5	P-4	P-3	P-2	P-1	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
1	+	+	-	+	+	-	-	-	-	-	-
					D	M										
2	-	-	-	-	-	-	-	+	-	-	-
				M	M	M	M	M	D	D						
3	+	+	+	+	-	-	-	-	-
					A	A	A	A								
4	...	+	+	+	-	+	+	-	+	+	-	-	-	-	-	-
					D			D			D	M			D	
5	+	+	+	+	+	-	-	-	-	-
								D				M				
6	+	+	+	+	+	+	-	-	-	-	-	-
			A	A	A	A	A	A								
7	-	+	+	+	+	+	+	+	-	-
8	+	+	+	-	+	-	-	-	-	-	-	-	...
							M				D				D	

^aFetuses were alive and normal at the time of necropsy of their dam unless noted otherwise. P = Principal fetus exposed to virus by experimental methods. C = Control fetus exposed to virus-free inoculum by experimental methods. + = Positive virus isolation. - = Negative virus isolation. D = Fetus that had recently died. M = Fetus that had died and was mummifying. A = Fetus that was alive but abnormal in appearance. ... = No fetus

noninfected littermates in litters 1 to 7, no difference was found. In litter 8, the CR mean was the same for live infected and live noninfected fetuses.

Discussion

In this study porcine fetuses from early through late gestation were susceptible to a direct inoculation of PRRSV; however, an age-related difference in the onset of mortality following inoculation may exist between the first and second half of gestation. Fetuses inoculated during the first half of gestation could replicate virus up to 31 days without severe, gross pathologic consequences. However, inoculation during the second half of gestation may result in death within days of exposure. In this method of fetal inoculation, we speculate that fetuses ingest the virus along with amniotic fluids and a viral infection is established. This route of infection is *unnatural*; nevertheless, it demonstrates that fetuses can support virus replication from 34 through 106 DG and that fetus-to-fetus and fetus-dam-fetus transmission may occur.

The clinical signs of late gestation reproductive failure have been reproduced with the North American prototype PRRSV isolate (SIRSV or ATCC-VR 2332). Transplacental infection did occur within days in all susceptible sows following oronasal exposure to virus at 93 DG (23). Transplacental infection did not occur by 7, 14, or 21 days in susceptible sows following oronasal exposure to PRRSV at or near 45 days of gestation; however, transplacental infection did occur in one litter within 65 days post exposure as evidenced by isolation of virus from two piglets at birth (22). In an analogous study, pregnant gilts were exposed to PRRSV by intravenous inoculation at or about 30, 50, 70, and 90 DG (107). Transplacental infection did not occur following inoculation of pregnant swine at 30 DG and only limited evidence was found for transmission following virus challenge at 50 and 70 DG while inoculation of gilts at 90 DG resulted in the typical signs of PRRS late-term reproductive failure. In a fetal susceptibility study, fetuses were exposed to PRRSV by intramuscular or intraamniotic exposure at or near 45 DG and when examined 4 and 11 days post inoculation the fetuses were alive, replicating virus, and normal in appearance (22). The PRRSV studies described here suggest that this isolate does not readily cause transplacental infections early in gestation, although fetuses apparently are susceptible to the virus at this time.

We completed a pilot study to determine the effects of direct inoculation of amniotic fluid with PRRSV in fetuses at 45, 65, or 85 DG (84). The gilts were euthanized seven days later and all fetuses inoculated with virus were alive, normal in appearance, and infected with virus. The *in utero* infection study was repeated with the fetuses of group 1 being examined 21-days-post exposure in an

attempt to cause fetal death with the virulent virus. Gross lesions were evident in each litter and 7 of 10 principal and 2 of 12 control fetuses had died in the litters exposed to virus during the second half of gestation, at 65 and 85 DG (Table II). All principal fetuses in litter 2 were dead and mummified when examined at 86 DG and based on condition and CR length they probably died one to two weeks prior to examination. In this litter, intrauterine spread of virus from principal to control uterine horn was presumed because a live control fetus (C-3) was positive for virus and two control fetuses nearest the uterine body had recently died. The principal fetuses infected during the first half of gestation in litter 3 were alive when examined at 66 DG although severely affected. The results of these litters suggested that a fetus may undergo a developmental change around 65 to 70 DG at which time it changes from being able to support virus replication without apparent gross lesions to an increased susceptibility and subsequent fetal death.

The absence of spontaneous abortions in this study following direct inoculation of amniotic fluids could be due to a variety of mechanisms, one of which could be the attenuation of virus by repeated passages in cell culture. The inoculum for litters 4, 5, and 6 of group 2 was prepared from the serum of fetus P-4, litter 3, a fetus that had a viremia of 10^6 TCID₅₀/mL. This inoculum was administered to fetuses in group 2 to test for an increase of virulence by *in vivo* passage. In addition, the fetuses in group 2 were exposed to virus at 49 DG and recovered 17 to 21 days later to test the observation that fetuses infected earlier in gestation could replicate the virus until about 65 to 70 DG at which time they would begin to succumb to the viral infection. Results from group 2 indicated that fetuses could replicate the virus to a very high serum titer and maintain life until they reached the projected stage of susceptibility at which time some of them had recently died and death appeared imminent for others. All principal fetuses were alive in litter 6 (necropsied at 70 DG) and replicating virus even though each one was very hemorrhagic and edematous with swollen, friable internal organs; this litter closely resembled litter 3 in size and appearance. We believe the virulence of the PRRSV isolate was unaffected by the single *in vivo* propagation and subsequent *in vitro* propagation of inoculum for group 2 and the additional *in vitro* propagation of virus for group 3 had no discernable effect.

Initially, the uterus of each gilt was flaccid when first exposed surgically; but following physical examination and manipulation, it would become firm. This phenomena made the identification and exact location of fetuses difficult to determine in the gravid uteri of group 3. In litter 7 at necropsy, the apparent remnants of two fetuses were identified in the control horn. The cause of death for these fetuses is unknown; but due to the scant amount of tissues found, the fetuses are thought to

have died around the time of surgery, implying that PRRSV probably was not involved. In this litter all principal fetuses were alive and normal in appearance including fetus P-1, the principal fetus that was mistakenly treated like a control. Virus was recovered from the serum of P-1 but not from its tissues, which was uncharacteristic for fetuses infected with virus for 21 days. The recovery of virus from the serum only and the low titer suggest this fetus may have been recently infected or had supported virus replication at a low rate. Virus was not recovered from one principal fetus, P-8, that was thought to have been inoculated at surgery. This fetus may not have been exposed to the inoculum or the concentration of virus inoculum ($10^{2.3}$ TCID₅₀) may have been near its threshold of infectivity. Since infected fetuses in litter 7 appeared healthy 21 days post exposure, gilt 8 was not euthanized until day 65 of gestation, or 31 days following exposure to virus.

In litter 8, two control fetuses had recently died. Virus was not recovered from them and their cause of death is unknown. It is possible that the fetuses were infected by a natural transuterine route since the dam apparently was exposed to virus, based on her seroconversion to PRRSV. In this study all gilts seroconverted to PRRSV following the *in utero* exposure of their fetuses and they were clinically normal throughout the study. The dam's exposure to virus may have been by contamination of maternal tissues or blood at the time of transuterine injection and/or their fetuses replicated the PRRSV and served as the source of infection. Virus was isolated from control fetuses in litters 2 and 4 indicating the fetuses had become infected probably by intrauterine spread of virus (Table II). Recent control fetus deaths had also occurred in these litters as well as in litter 8. These deaths may be the result of a recent PRRSV infection. Based on uterine position the control fetus deaths in litter 8 and possibly litter 4 could have occurred following a transuterine infection and not from intrauterine migration. This scenario suggests a low frequency of transuterine infections during the first half of gestation following infection of the dam.

The inability to recover virus from the two dead control fetuses in litter 8 is not surprising since PRRSV is apparently labile in autolytic tissues based upon positive isolation results: 33/34 (97%) live principal fetuses and only 1/12 (8%) dead principal fetuses. All of these fetuses were thought to have been exposed to the virus at the time of surgery. The single virus isolation from a dead fetus was from the thoracic fluids of a fetus that had recently died with minimal autolysis. One principal fetus, P-1 from litter 7, was not included in the above comparison because it was not exposed to virus experimentally but became infected later in gestation.

Although the actual mechanism is unknown, pathogenic viruses are thought to cross the maternal-fetal junction as free virus and/or as virus associated with a maternal cell that migrates

through the barrier. Once on the fetal side, the virus could infect placental tissues or enter the vasculature and/or the amnion and thus the fetus. The amniotic route may have a low probability, but we believe the fetus can become infected by this route as demonstrated in this study. It is not known if a difference exists between young and old fetuses in the mechanics of ingesting amniotic fluid and what influence it may have in the mortality reported in this study. We suggest that any potential difference would be inconsequential in establishing a lethal fetal infection.

Several possibilities may exist for the putative lack of clinical signs associated with PRRSV infection during the first half of gestation: 1) PRRSV-induced reproductive failure occurs at all stages of gestation and the signs observed during the first half of gestation are not equally reported; 2) differences in PRRSV virulence may exist between geographic regions; 3) transplacental infection of fetuses can occur at any time of gestation but fetuses do not succumb to viral infection until the second half of gestation; or 4) transplacental infection of fetuses does not occur until the second half of gestation.

Perhaps a unique pathogenesis exists for natural PRRSV infections in which susceptible fetuses are not readily infected during early gestation. Further investigation is warranted to test this hypothesis because of its potential impact on managing a breeding herd during a PRRS epizootic. Studies could be designed to evaluate the efficacy of immunizing susceptible sows with live virus or feedback of contaminated tissues prior to breeding or during early gestation. This immunization could protect sows in the herd from the reproductive failure that frequently, if not always, occurs following late gestation exposure.

Acknowledgements

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CHAPTER 4. PULMONARY LESIONS IN FETUSES EXPOSED *IN UTERO* TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

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The salient features of a porcine reproductive and respiratory syndrome (PRRS) epizootic include respiratory disease followed by reproductive failure. The respiratory component is usually recognized first in finishing pigs or the breeding herd as a mild, flu-like disease with a high morbidity for all ages and high mortality in younger pigs (80). In breeding herds, acute maternal reproductive failure often follows the onset of respiratory disease. It is characterized as a sudden increase in early farrowings, late-term abortions, stillborn and mummified fetuses, weak neonates with high mortality, an increase of late returns to estrus, and repeat breeders (80). No consistent microscopic lesions have been observed in fetal tissues from epizootics or following experimental infections of susceptible dams. The objective of this report is to describe fetal microscopic lesions associated with an experimental PRRS virus (PRRSV) infection.

Eight pregnant gilts underwent surgery at different stages of gestation: 2 at 34 days, 1 at 45 days, 3 at 49 days, 1 at 65 days, and 1 at 85 days gestation (Table 1) (85). All fetuses in one uterine horn (identified as principal fetuses) were exposed to a well characterized PRRSV isolate (11) by transuterine injection of virus into their amniotic fluids. All fetuses in the contralateral uterine horn (identified as control fetuses) were exposed to a sham inoculum in a similar fashion. The gilts were necropsied 17 to 31 days post surgery and their fetuses recovered for study. Virus isolation was attempted on sera from live fetuses, thoracic fluids from dead fetuses, and tissue composites from each fetus (85). Tissue samples for histopathology interpretation were collected from lung, kidney, liver, and spleen and placed in neutral buffered 10% formalin. Tissues were processed routinely, cut into 8 μ m sections and stained with hematoxylin and eosin. Sections of lung, liver and kidney were mounted in embedding media,^a frozen and sectioned on a cryostat. A fluorescein isothiocyanate labeled monoclonal antibody raised against the nucleocapsid protein of PRRSV (12) was used for direct staining of tissue sections as previously described (105).

Table 1. Virus isolation results for porcine fetuses.

Litter	Fetal age ^a	Principal		Control	
		Live	Dead	Live	Dead
1	34:55	7:8 ^b	0	0:2	0
2	34:65	4:4	0:1	0:5	0:2
3	45:66	4:4	0	0:5	0
4	49:66	5:5	0:2	2:5	0:3
5	49:68	5:5	0:1	0:3	0:1
6	49:70	6:6	0	0:6	0
7	65:86	0	0:5	1:4	0:2
8	85:106	3:3	1:2	0:6	0

^a Day of gestation principal fetuses inoculated: Day of gestation for necropsy of dam. ^b Number of fetuses infected with virus: Number of fetuses (dead or alive) in uterine horn.

Porcine reproductive and respiratory syndrome virus was recovered from 34 of 35 live principal fetuses and from 1 of 12 dead principal fetuses at the time of necropsy. Gross lesions in the dead fetuses consisted of mild to severe autolysis and mummification. Gross lesions were observed in some live principal fetuses which consisted of mild to severe cutaneous petechial hemorrhages and hemorrhagic and friable internal organs. In litter 7, all principal fetuses had died and were mummified at the time of necropsy. Two control fetuses located next to the uterine body had recently died and were beginning to mummify. Virus was recovered from the live control fetus adjacent to the dead control fetuses, implying that virus had spread from the principal to the control uterine horn. Lesions comparable with those of the principal fetuses were present in this live control fetus infected with virus. Histopathology was not attempted on any mummified fetuses; however, sections of lung tissue from all dead fetuses were examined for porcine parvovirus antigen by immunofluorescence as previously described (105). No porcine parvovirus antigen was observed in any dead fetus, moreover, no cytopathic agents were recovered from the dead fetuses other than the previously described PRRSV isolation in one dead fetus. The cause of death for the 11 fetuses for which no agent was isolated is unknown; however, we presume their deaths are the result of exposure to PRRSV and subsequent fetal infection. Litter 4 had 2 fetuses located next to the uterine body from which PRRSV was recovered. The location of the infected control fetuses in litters 4 and 7 suggest that intrauterine spread of virus probably occurred.

The most consistent and severe microscopic lesions were present in lung tissues from fetuses inoculated between 45 and 49 days of gestation; most notably litters 3 and 6 (Table 2). All principal fetuses from litters 3 and 6 had pulmonary lesions and 3 of 5 and 4 of 5 principal fetuses in litters 4

Table 2. Evaluation of lesions in porcine fetuses.

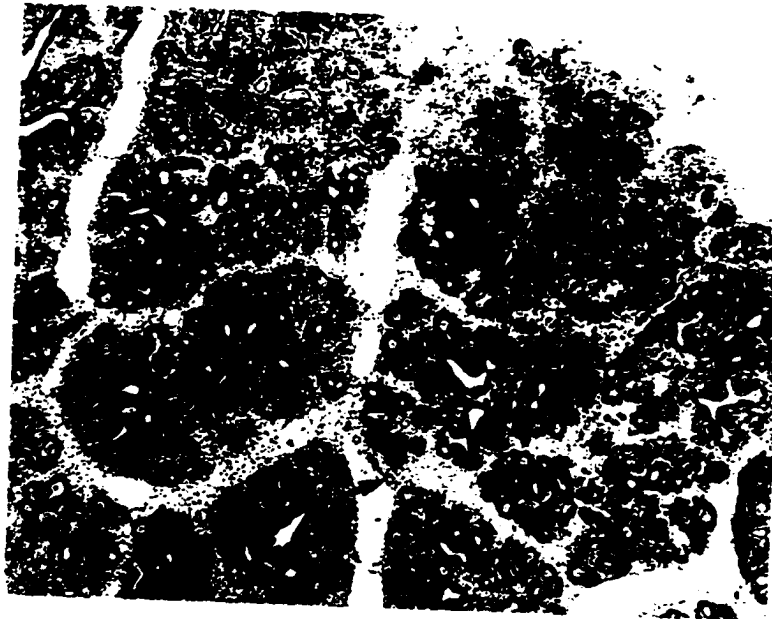
Litter	Fetal age ^a	Principal		Control		Lesion severity ^c
		Live	Dead	Live	Dead	
1	34:55	0:8 ^b	0	0:2	0	0
2	34:65	0:4	0:1	0:5	0:2	0
3	45:66	4:4	0	0:5	0	2.8
4	49:66	3:5	0:2	0:5	0:3	1.2
5	49:68	4:5	0:1	0:3	0:1	1.8
6	49:70	6:6	0	0:6	0	2.7
7	65:86	0	ND	1:4	0:2	0
8	85:106	0:3	0:2	0:6	0	0

^a Day of gestation principal fetuses inoculated: Day of gestation for necropsy of dam. ^b Number fetuses with lesions: Number of fetuses (dead or alive) in uterine horn. ^c Pulmonary lesions were scored individually for each pig on a scale of 0 to 4 (4 = most severe) and reported as the average value of affected pigs in litter, see text for details. ND = Not done, fetuses were mummified.

and 5, respectively, had lesions. The lesions were similar between pigs but differed in severity. The most severely affected were characterized by marked focally-extensive hemorrhage into the mesenchymal interstitium surrounding bronchial buds, large bronchi, and blood vessels (Fig 1). Within these areas of hemorrhage, lesions were also present in bronchial buds. There was marked segmental to circumferential necrosis of smooth muscle cells and mesenchymal spindle cells subjacent to the epithelial cells of bronchial buds (Fig 2). Many of these buds were moderately dilated and contained small amounts of necrotic cell debris. In some of these airways there was moderate, focally-extensive necrosis of the epithelium (Fig 3). These airways were often markedly distended and variably-filled by necrotic cell debris. Associated with the necrotic bronchial buds were moderate numbers of mononuclear cells within the peribronchial interstitium. There were no obvious organisms or inclusion bodies. Pulmonary lesions were scored, subjectively, with predetermined criteria: where 0 = minimal hemorrhage and congestion; 1 = mild, focally-extensive hemorrhage that was perivascular involving several blood vessels; 2 = moderate, focally-extensive hemorrhage that was perivascular and involving >5 blood vessels along with multifocal necrosis of elongate cells surrounding bronchial buds; 3 = marked, focally-extensive hemorrhage that was perivascular, interstitial, and intra-airway throughout the lesion and necrosis of elongate cells and epithelial cells of bronchial buds; 4 = severe hemorrhage throughout the section along with necrosis of bronchial bud epithelial cells and surrounding elongate cells. Replicating virus was identified in all litters indicating that fetuses could support virus replication from early gestation through term.

Fig 1. Lung. There is marked, focally-extensive hemorrhage, bronchial bud degeneration and necrosis (arrows demarcate the extent of the lesion). HE.

Fig 2. Lung. Bronchial bud in which there is necrosis of the smooth muscle cells surrounding the airway (arrow). There is marked congestion and hemorrhage. HE.



However, the lack of lesions in fetuses inoculated at times outside of 45 to 49 days of gestation suggest that there is a time of susceptibility during mid-gestation (Table 2).

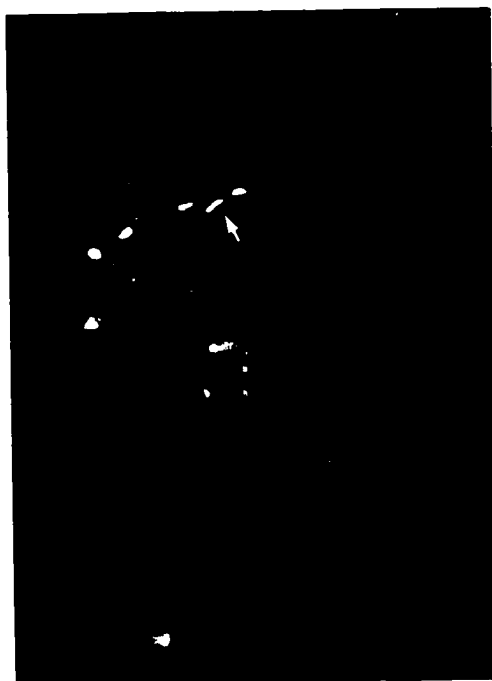
A less striking and consistent feature in fetuses from litters 3 to 6 was multifocal renal cortical hemorrhage. Multifocal and random renal glomeruli from many of these fetuses had dilated and congested glomerular capillaries and numerous red blood cells in the Bowman's space between parietal and visceral cells. In addition, there were mild multifocal areas of hemorrhage within the cortical interstitium. Similar areas of hemorrhage within both the glomeruli and interstitium were also present in control fetuses but to a lesser degree. Litters 3 to 6 had an average of 5.1 areas of renal hemorrhage per section of kidney in fetuses from inoculated horns, compared to 0.38 areas of hemorrhage per fetus from control horns. In addition, 18 of 22 fetuses from principal uterine horns that were alive at the time of necropsy had renal lesions and 4 of 20 fetuses from control uterine horns had lesions. Several spleens from fetuses of both principal and control uterine horns had expansion of the red pulp by blood.

Viral antigen was observed only in tissues of fetuses that were positive by virus isolation methods. Although specific cell types containing virus antigen could not be distinguished, antigen was observed in randomly distributed foci throughout the lung and liver tissue. In the kidney, foci of antigen were localized in the renal cortex. Antigen was most easily seen in sections of lung (Fig 4).

Diagnostic investigations of PRRS epizootics have not reported any consistent microscopic lesions in aborted fetuses or neonates (35, 135). Likewise, lesions have not been observed following experimental reproduction of PRRS maternal reproductive failure during late-term gestation (25). This report is the first description of fetal lesions associated with natural or experimental PRRSV infection. Generally, microscopic lesions associated with viral infection were in fetuses exposed to virus between 45 to 49 days of gestation and examined 17 to 21 days later. Fetuses exposed to virus during early gestation (about 34 days) had replicating virus and were normal in appearance when examined 21 and 31 days post exposure. In contrast, some fetuses exposed to virus during late gestation (65 and 85 days) had died and their deaths were attributed to PRRSV infection. In litter 7 (65 days), all principal fetuses were mummified when examined 21 days later. Based upon the extent of mummification, they were estimated to have been dead at least 7 to 10 days, implying they had survived the surgical procedure and probably had supported virus replication prior to death. In litter 8 (85 days), 2 fetuses exposed to virus were dead at the time of necropsy, one was undergoing mummification, and one had recently died. The remaining 3 principal fetuses were alive, had replicating virus, and were normal in appearance. Upon necropsy of the live principal fetuses,

Fig 3. Lung. Bronchial bud in which there is necrosis of epithelial cells. The lumen is moderately dilated and contains cell debris. HE.

Fig 4. Lung. Foci of antigen distributed throughout tissue (arrow).



varying amounts of thoracic fluid and edema of the abdominal viscera were observed. The gross lesions were not considered pathognomonic for PRRSV since similar lesions are frequently observed following experimental fetal infection in our laboratory with several virulent viruses. Their development is thought to be a normal consequence of fetal infection prior to death of the fetus. Litter 8 was examined during late gestation at approximately the time when clinical signs of PRRS epizootics are usually observed and it resembled descriptions of field cases based upon fetal death with concomitant lack of microscopic lesions.

This report suggests that fetuses during mid to late gestation are more susceptible to the lethal effects of a PRRSV infection. One possible mechanism for this putative window of increased susceptibility may be that a lethal event occurs when some cell population(s) become infected during normal ontogeny. This proposed cell type may be separate from the population(s) of cells that support viral replication during early gestation without obvious lesions. Studies are underway to test this hypothesis and other possibilities.

Acknowledgements

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Sources and manufacturers

- a. Tissue-Tek® O.C.T. Compound, Miles Inc., Elkhart, IN.

CHAPTER 5. EFFECT OF POST-COITAL INTRAUTERINE INOCULATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ON CONCEPTION IN GILTS

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Abstract

The effect of porcine reproductive and respiratory syndrome virus (PRRSV) on early gestation was investigated by exposing susceptible gilts to the virus shortly after they had been bred naturally. Gilts were exposed intrauterinely via an insemination pipette to either PRRSV (16 gilts) or a sham inoculum (23 gilts). On day 1 post exposure (PE), and on or about days 7, 14, and 30 PE, gilts were bled and serum was tested for PRRSV and homologous antibody. The pregnancy status of each gilt was determined on day 30 by ultrasound, and near or at term by either necropsy or allowing gilts to farrow naturally. All of the 16 gilts exposed to PRRSV became infected as evidenced by detecting PRRSV (7 gilts) and homologous antibody (16 gilts) in their serum, whereas all of the 23 gilts exposed to sham inoculum remained free of both virus and antibody throughout the study. Although there was a difference in the conception rates of infected and noninfected gilts (10/16 vs 19/23), the difference was not statistically significant ($P = <0.05$). Moreover, the mean numbers of live fetuses or pigs per litter of infected and noninfected gilts were similar (9.7 vs 9.3). These results suggest that intrauterine infection of susceptible pigs with PRRSV at or near the time of conception may have little or no effect on reproductive performance.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first observed in the United States during the late 1980's as sporadic epizootics of respiratory disease in swine herds followed by maternal reproductive failure (80). This new syndrome spread rapidly throughout the major swine producing regions of the United States and into Canada resulting in significant losses for the swine industry. It was first reported in Europe in late 1990 and within months, PRRS was reported

throughout Western Europe and in Great Britain despite strict, conventional quarantine methods. The causative agent, the PRRS virus (PRRSV), was identified in 1991 and the virus is now thought to have a world wide distribution (187).

The maternal reproductive failure was characterized by an increase in late-term abortions and early farrowings with mummified fetuses, stillborn and weak piglets, late returns to oestrus, and repeat breeders resulting in a significant reduction of pigs weaned per sow. From the beginning, pig-to-pig contact was assumed to be a major route of virus transmission; however, it did not account for the apparent transmission of PRRSV over great distances and the infection of swine located in strict biosecurity farms. Field investigations have implicated the use of fresh semen from recently infected boars and aerosol transmission of virus as potential modes of transmission that circumvent quarantine procedures and distance.

Recent reports have indicated that boars can shed PRRSV in their semen following exposure to virus, (26, 168, 196) thus supporting earlier hypotheses that boar studs played a role in transmitting the disease by the distribution of contaminated semen (48, 147, 196). Field exposure of boars to PRRSV may result in a variety of clinical manifestations from subclinical infections to varying degrees of anorexia, lethargy and loss of libido (49, 73). Semen quality is thought to decrease following field infections although limited quantitative data is available (42, 49). Experimental infection of boars has resulted in few if any clinical signs; in two studies semen quality remained within normal limits except for a decrease in volume (168, 196) and in a third study, a decrease in motility and morphologic changes were observed (143). Experimental data is limited for the consequences of PRRSV exposure on conception. Infertility associated with PRRSV was reported in two studies in which susceptible gilts were exposed to virus-contaminated semen (168, 196). This suggests the decrease in conception rates described during PRRS epizootics may be attributed to the presence of virus in the semen, which may affect the conceptus, uterine environment, or semen quality.

The mechanism of putative PRRSV infertility in the sow could be due to one or more of the following: 1) semen quality; 2) altered uterine environment; 3) complications involving placentation; 4) direct effects on the unfertilized egg; or 5) direct effects on the conceptus. The following study was undertaken to test the effects of PRRSV on conception in swine by exposing susceptible gilts to an intrauterine virus inoculation following natural breeding.

Materials and methods

Experimental design -- Thirty-nine gilts were purchased from a farm free of PRRSV-specific antibody and clinical signs of reproductive failure. The gilts were removed from the finishing herd at approximately 110 kg of body weight and exposed to boars. The gilts were each bred on their second and third day of standing heat or 0 and 1 day of gestation (DG), respectively. Following the second breeding or refusal of boar on 1 DG, the gilts were transported to our laboratory, randomly assigned to one of two groups, and inoculated with a commercial vaccine for 5 serovars of leptospira, porcine parvovirus and Erysipelothrix rhusiopathiae. Group 1 consisted of 16 gilts exposed through an artificial insemination pipette to an intrauterine inoculation of 5 ml of a PRRSV inoculum [Titer = $10^{5.7}$ median cell culture infective doses (CCID₅₀)/ml] upon arrival and housed for 21 days in isolation facilities with one to three gilts per room. Following isolation, the virus-exposed gilts were housed as a group in an outdoor facility. Group 2 consisted of 23 gilts exposed to 5 ml of a sham inoculum in a like fashion and housed as a group in a separate outdoor facility. The sham inoculum was prepared from medium harvested from mock-infected cell cultures. Blood samples were collected at selected times: 1 DG, 7 to 9 DG, 14 to 16 DG and 30 to 32 DG and serum was tested for the presence of PRRSV and virus-specific antibody(106). Gilts were examined with a handheld ultrasound device every other day beginning at 22 DG and their pregnancy status was determined at 30 DG. In addition, gilts in group 2 were checked daily with a boar for a return to estrus. Gilts were either euthanized and necropsied on or about 112 DG, or allowed to farrow. The number of fetuses/piglets in each litter was determined and the mean litter size for each group was calculated.

Virus -- Strain NADC-8 of PRRSV was used to infect gilts during this study. It had been isolated in our laboratory by exposing a culture of porcine alveolar macrophages to serum of a 2-day-old, moribund piglet from a naturally occurring case of PRRS in Iowa (all of the piglet's littermates had either died in utero or were stillborn). It was subsequently propagated (passaged) once in a 4-week-old pig and then three times in MARC-145 cells. The infectivity titer of the stock virus (i.e., the third passage in MARC-145 cells) was $10^{5.7}$ CCID₅₀/mL.

Statistics -- The conception rate was determined by the following method for each group: per cent pregnant = the number of gilts pregnant at 30 DG/the number of gilts bred x 100. Differences in conception rate were analyzed by chi square analysis. The mean litter size for groups 1 and 2 were analyzed by Student's t-test.

Results

All gilts were clinically normal throughout the duration of the study. Neither PRRSV nor PRRSV-specific antibody were detected in the sera of group 2 throughout the experiment. In group 1, virus was identified in the sera from 7 of the 16 gilts tested 7 to 9 DG (6 to 8 days post intrauterine inoculation of virus); however, virus was not detected in the sera of any other gilts at any other time. Virus specific antibody was detected in 2 gilts at 7 and 9 DG, respectively, and in all 16 gilts by 14 to 16 DG. In group 1, 10 of 16 gilts (63 per cent) were pregnant compared to 19 of 23 gilts (83 per cent) in group 2 at 30 DG resulting in an insignificant difference ($P = 0.157$). The mean litter size for groups 1 (9.7 fetuses/litter) and 2 (9.2 fetuses/litter) were similar ($P = 0.545$). All fetuses in group 1 were normal in appearance and neither PRRSV nor PRRSV-specific antibody were detected in their sera.

Discussion

Intrauterine exposure of PRRSV to susceptible gilts around the time of conception resulted in an insignificant decrease in conception rate. All gilts deemed pregnant at 30 days of gestation maintained their pregnancy throughout the study. No difference in litter size was found between litters of dams exposed to virus at the time of conception and the litters of sham inoculated controls. Moreover, all litters in group 1 were normal in appearance when examined on or about 112 DG; the time at which the gilts were euthanized. This experiment demonstrated that swine are susceptible to an intrauterine inoculation of virus as evidenced by the detection of virus in the serum of 7 of 16 gilts and virus-specific antibody in 16 of 16 gilts approximately 1 and 2 weeks post inoculation, respectively. The experiment was designed to evaluate the contribution of the dam, and thus indirectly the boar, to PRRSV-associated infertility. Presumably the direct inoculation of virus would have resulted in a significant decrease in conception if PRRSV-associated infertility is dependent on viral effects in the dam or conceptus. A significant decrease in conception did not occur which suggests: 1) that PRRSV may play a limited role in infertility, or 2) the virus-associated infertility is dependent on strain variation, or 3) the boar may be the major contributor to PRRSV-associated infertility.

Although this study suggests direct intrauterine inoculation of virus has little effect on conception, the slight decrease in conception rate may represent the actual influences of PRRSV since field reports indicate variable reductions in conception rates following PRRS epizootics(73, 80). If this is true, then the variable effect may be due to virus strains or involve unknown

mechanisms involving a temporal relationship between infection of the dam and the time of conception which results in an altered uterine environment that is detrimental to development of the conceptus. In this study no relationship was discernible between the gilts that did not conceive in group 1 and their ability to develop antibody or the detection of virus in their sera.

The 10 gilts that did not conceive were naturally bred to a boar; 4 of the 6 gilts in group 1 and all 4 gilts in group 2 conceived after breeding. The 2 open gilts from group 1 did not conceive following 4 additional breedings each (unpublished observations). We believe these 2 gilts from group 1 to be infertile and suggest this infertility is not associated with PRRSV. If these gilts are not included in group 1, then 10 out of 14 gilts conceived for a conception rate of 71 per cent, a rate that is similar to the control group.

Two preliminary studies have reported infertility in susceptible gilts following insemination with virus-contaminated semen collected from boars recently infected with PRRSV (168, 196). These reports in conjunction with the present study suggest that if PRRSV causes infertility then the quality of the semen from the boar may play a significant role. Field reports and preliminary experimental studies indicate that PRRSV infections of the boar result in variable effects on the common parameters of semen quality, i.e., motility, morphology, concentration and volume of ejaculate. Further investigation is needed to determine the acute and long term effects of PRRSV infections in boars and their subsequent effects on fertility.

Acknowledgements

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CHAPTER 6. GROSS AND MICROSCOPIC LESIONS IN PORCINE FETUSES INFECTED WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

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Abstract

Diagnosis of porcine reproductive and respiratory syndrome (PRRS) virus-induced reproductive failure in swine is difficult because of the rapid inactivation of virus in fetuses that have died prior to abortion or farrowing. In this report, we describe gross and microscopic lesions of diagnostic value found in fetuses transplacentally infected with PRRS virus (PRRSV) during late gestation. Seven sows free of PRRSV-specific antibody and one sow (#8) that had been previously infected with PRRSV were oronasally exposed to a PRRSV inoculum at or about 90 days of gestation (DG). One control sow (#9) was oronasally exposed to a sham inoculum at 90 DG. Sows were euthanized 21 days-post-exposure and fetuses were tested for virus. Transplacental infection was detected in litters 1 through 7 and gross lesions of the umbilical cord were observed in some fetuses in 6 of the 7 litters. No transplacental infection or fetal lesions were found in litters 8 and 9. The gross lesions in the umbilical cords ranged from segmental hemorrhagic areas 1 to 2 cm in length to a full length involvement of the cord which was grossly distended with frank hemorrhage. All live fetuses that had gross lesions in their umbilical cord were viremic and a necrotizing umbilical arteritis with periarterial hemorrhage was found by histopathological examination. This was the most consistent microscopic lesion in fetuses infected with PRRSV. Sows 1 through 7 had endometritis and myometritis of varying degrees suggesting PRRSV may induce these lesions as well. This study indicates careful gross and microscopic examination of the umbilical cord may aid in the diagnosis of PRRSV-induced reproductive failure.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease of swine characterized by late-term, maternal reproductive failure and by respiratory disease in growing pigs. Abortions are occasionally reported during early to mid-gestation (73), and reduced conception rates are frequently associated with PRRS epizootics (73, 80). However, most field reports and epidemiologic studies describe late-term reproductive failure, i.e., abortions, late-term in utero fetal death, mummies, stillborn, and weak piglets, as the salient clinical signs for the reproductive disease (30, 35, 44, 73, 80, 135, 164, 175). The respiratory disease has a high morbidity for swine of all ages and is more severe in young pigs; it is typified by labored breathing and the development of a characteristic interstitial pneumonia (60, 61). PRRS virus (PRRSV) is the etiologic agent and is tentatively classified in the proposed family, Arteriviridae, which includes equine arteritis virus, lactate dehydrogenase-elevating virus and simian hemorrhagic fever virus (31, 117).

Diagnosis of PRRS reproductive failure is typically based on indirect evidence like clinical signs and serology, e.g., seroconversion of the sow herd, rise in antibody titer in paired sera collected from sows, or virus detected in piglets in the farrowing house or nursery. Collecting direct evidence by detecting virus in aborted or stillborn fetuses is difficult because of the rapid inactivation of virus in fetuses that have died prior to abortion or farrowing. Consistent lesions have not been found in tissues of aborted or stillborn fetuses collected from field cases or from experimentally induced, late-term reproductive failure (25, 44, 164); however, lesions have been observed in the uterus following natural and experimental infections of the sow. Multiple foci of lymphoplasmacytic inflammation have been observed in the myometrium of some sows experimentally infected with PRRSV.¹ In a separate study, myometritis, endometritis and placentitis of the maternal placenta were observed in experimentally and naturally infected sows (164).

During the course of several experiments involving infection of pregnant swine during late gestation with PRRSV, we have observed a pattern of characteristic gross lesions that develop in transplacentally-infected fetuses. In this report, we describe gross and microscopic lesions found in fetuses transplacentally infected with PRRSV during late gestation.

Materials and Methods

Virus -- The NADC-8 strain and the Lelystad strain of PRRSV were used in this study. The NADC-8 strain had been isolated in our laboratory by exposing a culture of porcine alveolar macrophages to serum of a 2-day-old, moribund piglet from a naturally occurring case of PRRS in Iowa (all of the

piglet's littermates had either died in utero or were stillborn). A 4-week-old piglet was oronasally inoculated with 2 ml of medium collected from the macrophage culture. Serum was harvested from the piglet 7 days post-inoculation and was used to inoculate a PRRSV permissive cell line, MARC-145 (81). The virus was subsequently propagated (passaged) 3 times in cell culture and the medium harvested to produce a stock virus which was aliquoted into 5 ml volumes and stored at -80 C. The infectivity titer of the stock virus (i.e., the 3rd passage in MARC-145 cells) was approximately 1×10^6 CCID₅₀/ml.

The Lelystad virus inoculum was prepared in the following manner. A 5 ml aliquot of cell culture medium harvested from alveolar macrophages infected with Lelystad virus was obtained (J. B. Katz of the National Veterinary Services Laboratory, Ames, IA) and oronasally administered to a sow at 90 days of gestation (DG). She was euthanized 21 days-post-exposure (DPE) and serum was harvested from transplacentally-infected fetuses. The fetal serum was used to inoculate a monolayer of MARC-145 cells and the progeny virus was propagated twice more to prepare a virus stock. The virus stock was adjusted to an approximate titer of 1×10^6 CCID₅₀/ml and aliquoted into 5 ml volumes and stored at - 80 C.

A sham inoculum was prepared from cell culture medium recovered from flasks of MARC-145 cells treated similarly as the virus infected flasks; the inoculum was aliquoted and stored at -80 C until use.

Experimental design -- Seven sows determined free of PRRSV-specific antibody by indirect immunofluorescence microscopy (108) and one sow (#8) that had been previously infected with the NADC-8 strain of PRRSV 567 days earlier were oronasally exposed to either the NADC-8 (sows 1 to 6 and 8) or Lelystad (sow 7) strain of PRRSV at or about 90 DG (Table 1). One sow (#9) free of PRRSV-specific antibody was oronasally exposed to a sham inoculum at 90 DG. All sows were housed in isolation facilities until 21 DPE at which time the animals were euthanized with an intravenous overdose of pentobarbital. Fetuses were removed from the uterus and their relative uterine position recorded, e.g., a fetus located next to the ovarian tip of the left uterine horn would be identified as L-1 and the next fetus as L-2, etc. Fetuses were classified as dead, live-abnormal or live-normal based on gross lesions and a detectable pulse in their umbilical cord. Dead fetuses were presumed to have been dead ≤ 1 day if mild to no autolysis was present, dead 1 to 2 days if moderate autolysis (fetal tissues becoming reddened, gelatinous, edematous) was present, or dead ≥ 3 days if severe autolysis (fetal tissues becoming brown to grayish in color, eyes sunken, and tissues could

easily be pulled apart) was present. Live fetuses were classified as abnormal or normal in appearance based on presence of gross lesions defined below. Depending on fetal condition, a tissue composite (lung, liver, spleen, kidney and heart) and blood or thoracic fluid was collected.

Serology -- Sow sera were examined for PRRSV-specific antibody by indirect immunofluorescence microscopy as previously described (108).

Virus Isolation -- Fetal fluids and tissues were tested for virus as previously described (108) and results reported as positive or negative based on isolation of virus from fluids, tissues or both.

Histopathology -- Some of the fetuses from each litter were selected for microscopic examination based on normal and abnormal gross appearances. Sections of fetal lung, liver, spleen, kidney, heart, umbilical cord, and usually, a full-thickness section of the uterus and placenta corresponding to an individual fetus were collected. Tissues were fixed by immersion in 10% neutral buffered formalin, processed in an automated tissue processor, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Tissues were examined in a blinded fashion.

Results

Sows 1 through 7 developed PRRSV-specific antibodies following primary exposure to virus. The antibody titer of sow 8, the sow that had previously been exposed to PRRSV, did not change following a second exposure to PRRSV (data not shown). PRRSV-specific antibody was not detected in sera of sow 9, the sham inoculated control sow. Transplacental infection and fetal gross lesions were detected in each of the litters 1 through 7. No transplacental infection or gross lesions were identified in litters 8 and 9. A total of 87 fetuses were in the 7 transplacentally infected litters; 36 were considered normal, 24 live-abnormal, 8 dead \leq 1 day, 6 dead 1-2 days, and 13 dead \geq 3 days (Table 1). Virus was recovered from 16 (44%) normal, 23 (96%) abnormal and 4 (50%) fetuses that had been dead \leq 1 day (Table 1).

Litter 1 consisted of 7 fetuses in the right uterine horn (R-1 to R-7) and 6 in the left (L-1 to L-6). Two of 13 fetuses were live-normal and 2 were live-abnormal in appearance. The remaining 9 were dead; 2 of which appeared to have died within hours of the sow's death, one estimated to have been dead 1 to 2 days, and the remainder estimated to have been dead greater than 3 days (Table 1). In our laboratory, this presentation of normal, abnormal, and dead fetuses is typical in the experimental

Table 1. PRRS virus isolation and gross observations of fetuses examined at or about 111 days of gestation.

Sow	Virus ^b	No. Fetuses ^a						
		Live			Dead			Total
		Normal	Abnormal ^c	Total	≤1 day	1-2 days	≥3 days	
1	NADC-8	2(2)	2(2)	4(4)	2(1)	1(0)	6(0)	9(1)
2	NADC-8	5(1)	5(5)	10(6)	0	2(0)	2(0)	4(0)
3	NADC-8	2(1)	8(7)	10(8)	0	1(0)	0	1(0)
4	NADC-8	8(8)	3(3)	11(11)	1(0)	2(0)	0	3(0)
5	NADC-8	4(0)	4(4)	8(4)	3(2)	0	4(0)	7(2)
6	NADC-8	6(2)	2(2)	8(4)	1(1)	0	1(0)	2(1)
7	Lelystad	8(1)	1(1)	9(2)	1(0)	0	0	1(0)
	Total	36(16)	24(23)	60(39)	8(4)	6(0)	13(0)	27(4)
8	NADC-8	15	0	15(0)	0	0	0	0
9	Sham	13	0	13(0)	0	0	0	0

^a Number of fetuses positive for virus given in parentheses. ^b Isolate of PRRSV oronasally administered to sow at or about 90 days of gestation. ^c Live fetuses abnormal in appearance due to gross lesions of the umbilical cord and/or internal organs.

model described above. Litter 1 will be considered the prototypical PRRSV infected litter and its gross and microscopic lesions will be described in detail.

In the right uterine horn fetus R-1 was normal in appearance and positive for virus. Virus was isolated from fetus R-2, a live-abnormal fetus which was covered with a mixture of meconium and amniotic fluids. The mixture did not contain formed meconium; instead, the meconium was worked into a thick, sticky substance which was adhered to the skin. The meconium/amniotic fluid mixture was stained with fresh blood. The fetus was washed off with water and it appeared normal in appearance except for cutaneous, petechial hemorrhages. Its umbilical cord was 2-3 times normal size and contained hemorrhages which were circumferential and involved most of the cords length (Fig 1). Fetuses R-3, 4, 5 and 7 were estimated to have been dead ≥ 3 days and extensive autolysis precluded any assessment of vital changes in the fetuses or their umbilical cords. Fetus R-6 was estimated to have been dead 1 to 2 days, it was slightly discolored and its umbilical cord was 2-3 times normal size with hemorrhages distributed throughout the cord.

In the left uterine horn fetuses L-1 and L-2 were estimated to have been dead ≥ 3 days. Fetus L-3 was normal in appearance and positive for virus. The internal organs of fetus L-4 appeared slightly

Figure 1. Umbilical cords collected from fetuses in litter 1 at 111 days of gestation. Fetus R1 was alive and appeared normal at the time of the sow's death; no gross lesions were observed in its umbilical cord. Fetus L5 was alive and appeared normal except for its umbilical cord. Fetus R2 was alive; however, it had cutaneous petechial hemorrhages and was covered with a blood-stained mixture of meconium and amniotic fluid. Fetus L4 was thought to have died shortly before the sow's death, and fetus L6 was thought to have been dead < 1 day. Umbilical cords from fetuses L5, R2, L4, and L6 contained gross hemorrhages. PRRSV was isolated from fetuses R1, L5, R2, and L4.

L-6



L-4



R-2



L-5



R-1



swollen and autolyzed, a clear effusion consisting of 10-15 ml was found in the thoracic cavity; this fetus was thought to have died shortly before euthanasia of the sow. Its umbilical cord contained segmental hemorrhages 2-5 cm in length. Fetus L-5 was considered live-abnormal because of several foci of hemorrhages in its umbilical cord which were less severe than R-2, otherwise the fetus was normal in appearance. Fetus L-6 was dead and also covered with a bloody meconium/amniotic fluid mixture, its internal organs appeared to have more postmortem autolysis than L-4 and a clear to serosanguineous effusion was in the thoracic cavity; this fetus was estimated to have been dead less than 1 day. Its umbilical cord was dark and distended 2-3 times normal size with blood. The placenta associated with L-6 was beginning to separate from the endometrium and contained scattered, paintbrush-like hemorrhages. Virus was isolated from L-4 and L-5, but not from L-6 or the other dead fetuses.

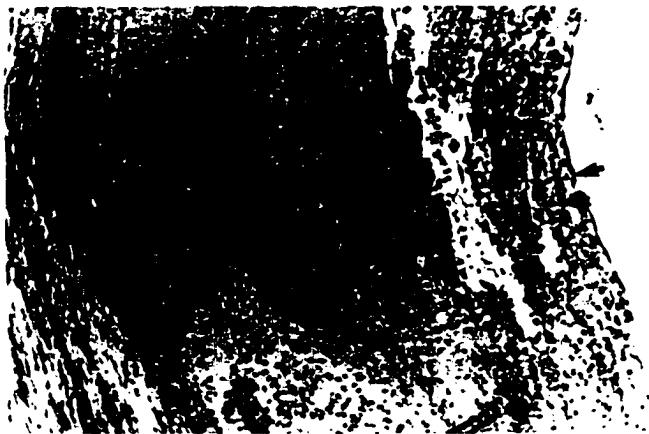
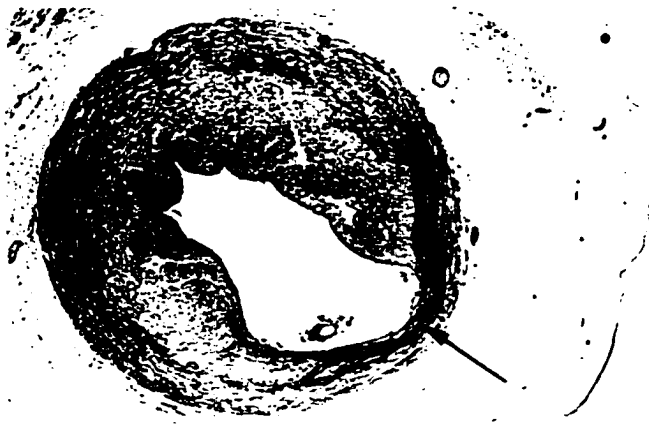
Six fetuses (R-1, R-2, L-3, L-4, L-5, L-6) representing normal (R-1, L-3), live-abnormal (R-2, L-5), and recently dead (L-4, L-6) fetuses were selected for microscopic examination from litter 1; the fetal lesions will be individually described followed by a summary of lesions from litters 1 through 7 (Table 2). Examination of internal organs from fetus R-1 revealed normal lung, heart, and liver; however, mild multifocal renal cortical hemorrhage was seen. Mild lymphoplasmacytic and histiocytic infiltrates within and around small capillaries at periphery of the tunica adventitia of umbilical arteries were observed.

Fetus R-2 had a mild multifocal perivascular lymphoplasmacytic myocarditis and a mild periportal and subcapsular lymphoplasmacytic, histiocytic, and neutrophilic hepatitis. In 5 of 5 sections of the umbilical cord examined, one or both of the umbilical arteries had a severe arteritis characterized by loss of endothelial lining and necrosis of the tunica intima and media with infiltration by macrophages and neutrophils. There was marked hemorrhage extending into the tunica adventitia and surrounding mucous connective tissues. There were sometimes pockets or clefts filled with red blood cells in the outer tunica media.

Fetus L-3 had normal lung, heart, liver, kidney, and spleen. Four of 5 sections of umbilical cord appeared normal and in 1 section a 2 to 4 cell layer thick lymphoplasmacytic and histiocytic infiltrate was seen in the subendothelium involving about one-half of the circumference of both umbilical arteries.

Fetus L-4 was thought to have died shortly before the death of the sow; its tissues were mildly autolyzed. Mild multifocal hemorrhage and congestion were observed in the kidney. Three of 5

Figure 2. Cross section of umbilical cords from fetuses transplacentally infected with PRRSV following oronasal inoculation of their dam 21 days earlier. **A.** Umbilical cords have moderate to severe hemorrhage into the connective tissue surrounding the umbilical arteries and vein. Umbilical arteries have segmental areas of necrosis of the tunica intima and tunica media (arrows). HE. **B.** Segmental necrosis and collapse of the tunica intima and tunica media is present in the umbilical artery (arrow). HE. **C.** Umbilical artery has marked necrotizing and fibrinosuppurative arteritis with hemorrhage into the tunica adventitia (arrow). Patches of endothelium remain intact (arrowhead). HE.



sections of the umbilical cord appeared normal while 2 sections contained severe lesions comparable to fetus R-2.

Mild, multifocal periportal mixed inflammation was present in the liver of L-5. Moderate hemorrhage and mild lymphohistiocytic inflammation was present in 1 of 5 sections of umbilical artery and severe periarterial hemorrhage was present in 2 of the remaining sections.

The fetal tissues of L-6 were moderately autolyzed, the lung, heart, liver, kidney and spleen appeared normal. A severe segmental arteritis was observed in the umbilical cord; it was characterized by swelling and loss of endothelium with marked necrosis of and hemorrhage into tunica intima and tunica media. Collagenous connective tissue had become coagulated and collapsed. Moderate-to-severe tunica adventitial and perivascular hemorrhage was present in 2 of 4 sections observed.

The uterine tissue associated with all 6 fetuses frequently had mild multifocal perivascular lymphoplasmacytic and histiocytic myometritis and a moderate multifocal nonsuppurative perivascular endometritis. Microseparation of maternal and fetal placental epithelium was rarely seen; sometimes it contained proteinaceous fluid.

The gross and microscopic lesions observed in litters 2 to 7 were similar to litter 1. In addition to lesions already reported in litter 1, gross lesions frequently associated with fetuses in other litters experimentally infected with PRRSV included edema located around the pancreas, kidney, and in the mesentery of the spiral colon. The edema was sometimes gelatinous around the kidney and the loops of the spiral colon were sometimes separated as much as 1 cm. A clear effusion was seen in the thoracic and abdominal cavities.

Microscopic lesions observed in fetal tissues from the 7 transplacentally-infected litters are summarized in Table 2. Fifteen out of 45 umbilical cords examined had microscopic lesions ranging from moderate-to-severe, segmental-to-circumferential, necrosuppurative and lymphohistiocytic arteritis with marked transmural and periarterial hemorrhage. Endothelial lining was often swollen or missing and subendothelial lymphohistiocytic aggregates were common (Fig 2). Thirteen of 53 fetal lungs examined contained microscopic lesions consisting of mild-to-moderate, multifocal-to-diffuse, histiocytic and proliferative interstitial pneumonia characterized by septal infiltration by mononuclear cells, hypertrophy and hyperplasia of type 2 pneumocytes, and accumulation of a small amount of mixed alveolar exudate. Multifocal hemorrhage was less consistent. A few pigs had mild segmental necrotizing and lymphohistiocytic pulmonary arteritis. Mild-to-moderate, periportal and subcapsular, lymphohistiocytic, suppurative, and less often eosinophilic, hepatitis was seen in 4 of 53

Table 2. Results ^a of microscopic examination of porcine fetuses and associated uterine/placental tissues.

Litter	Umbilical cord	Lung	Liver	Heart	Kidney	Spleen	Myo- metrium	Endo- metrium	Placenta
1	6/6	0/6	2/6	1/6	0/6	0/5	5/6	4/6	0/6
2	0/4	0/7	0/7	ND	0/7	0/7	4/6	4/6	0/4
3	0/9	3/9	0/8	ND	0/8	0/9	9/9	7/9	2/9
4	3/9	2/8	0/9	0/3	0/9	0/8	2/8	2/8	0/9
5	0/11	1/11	0/11	ND	0/11	0/11	8/11	6/11	0/10
6	3/3	5/9	2/9	0/9	0/9	0/9	ND	ND	ND
7	3/3	2/3	0/3	2/3	0/3	0/2	1/3	2/3	0/3
Total	15/45	13/53	4/53	3/21	0/53	0/51	29/43	25/43	2/41
8	0/8	0/8	0/8	ND	0/8	0/8	0/8	0/8	1/8
9	0/8	0/8	0/8	ND	0/8	0/8	0/8	0/8	0/8
Total	0	0	0	0	0	0	0	0	1/16

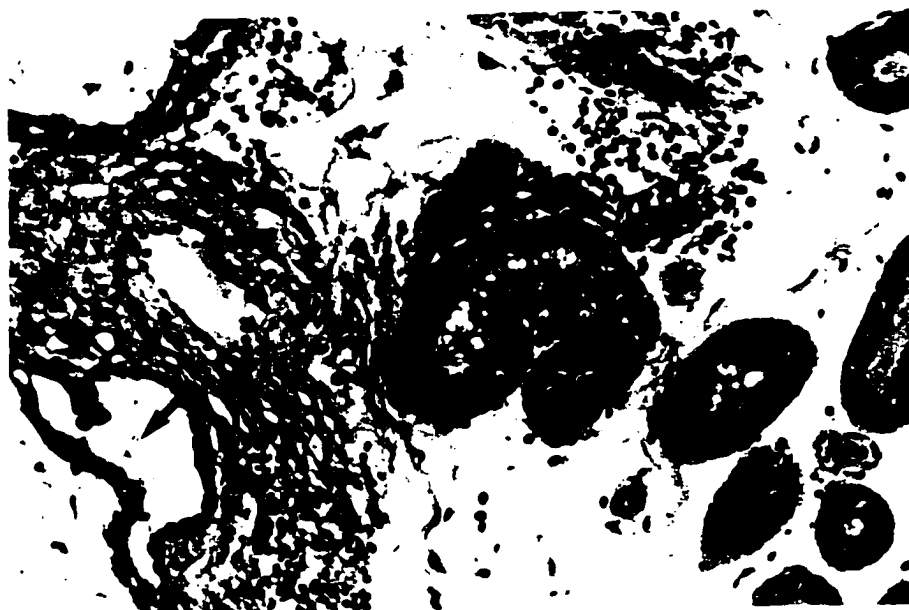
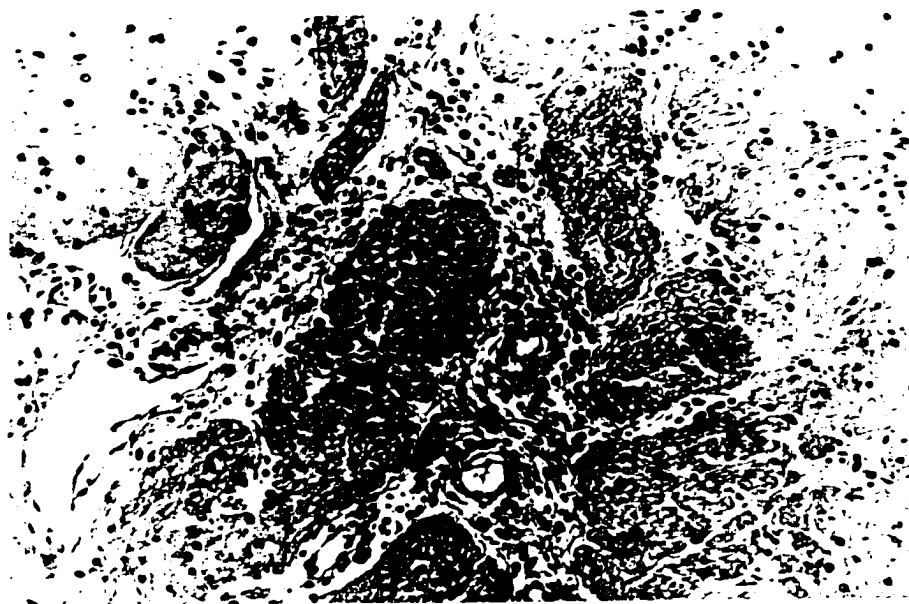
^a Number of fetuses with lesions/number of fetuses examined. ND = not done.

fetal livers examined. Lesions were observed in 3 of 21 hearts examined and were characterized by mild, multifocal, perivascular, lymphohistiocytic myocarditis. Multifocal myocardial hemorrhage was less consistent.

Lesions were found in 25 of 43 sections of endometrium consisting of mild to severe, usually perivascular, pleocellular endometritis. Endometrial edema was common. Mild-to-moderate, multifocal, microseparation of uterine and fetal placental epithelium was seen. Proteinaceous fluid commonly collected in this cleft, however inflammation here was rare suggesting that this separation may be an artifact (Fig 3). Twenty-nine of 43 sections of myometrium had lesions characterized by mild-to-severe, usually perivascular, pleocellular myometritis. Edema between smooth muscle bundles was common (Fig 3). Placental lesions were only seen in tissues associated with one fetus in litter 8 and two fetuses in litter 3. The lesions consisted of moderate, diffuse, lymphoplasmacytic placentitis with mild multifocal arteritis.

Sow 6 aborted at 111 DG, or 20 DPE and her litter consisted of 9 pigs and 1 fetus beginning to mummify. Five of the pigs were alive and very weak when found, 4 of these had suckled and virus was isolated from 1. The remaining 4 pigs were dead; 1 was stillborn and wrapped in fetal membranes, the other 3 pigs were born alive and had died, virus was recovered from all 4 of these

Figure 3. Uterus of gilts oronasally inoculated with PRRSV 21 days earlier. **A.** Moderate lymphoplasmacytic and histiocytic perivascular myometritis with mild interstitial edema is present. HE. **B.** Moderate multifocal and perivascular lymphoplasmacytic and histiocytic endometritis with microseparation (arrowhead) of maternal and fetal placental epithelium is present. HE.



dead pigs. Each of the dead fetuses had 6 to 10 cm of their umbilical cord attached, 3 contained gross hemorrhages (including the stillborn pig) similar to that of infected fetuses recovered following necropsy of the other sows prior to farrowing. Segmental necrosuppurative arteritis with marked hemorrhage throughout the wall of one or both umbilical arteries was present in each of the 3 cords. The live pigs had dried, dark brown, shrunken umbilical cords which were not examined microscopically. Examination of the placenta revealed stumps of several umbilical cords that appeared to contain hemorrhages similar to gross lesions seen in umbilical cords of PRRSV-infected fetuses; characteristic microscopic lesions were present in these cords.

For this report, litters 8 (seropositive sow) and 9 (sham infected controls) will be considered as non-infected control litters which contained 15 and 13 live-normal fetuses, respectively; virus was not isolated from either litter or from either sow. A moderate lymphoplasmacytic placentitis with mild multifocal arteritis was found in the placenta associated with one fetus in litter 8, otherwise the remaining uterine/placental tissues and fetuses appeared normal. No microscopic lesions were found in any of the fetuses or the uterine/placental tissues examined from sow 9.

Discussion

In this report we describe gross and microscopic lesions in fetuses following experimental infection of the dam with PRRSV during late gestation. The example of a "field case-like" late-term abortion, i.e. litter 6, demonstrates umbilical cord lesions could be found in the field and these tissues may be submitted to diagnostic laboratories as an aid in the diagnosis of PRRSV-induced maternal reproductive failure. Moreover, the gross and microscopic lesions reported here may offer insight into the pathogenesis of fetal death following PRRSV infection of the sow.

The experimental infection of sows at or about 90 DG and the examination of their litters 21 DPE has allowed us to examine a spectrum of lesions leading to fetal death following transplacental infection. In general, an infected litter would consist of fetuses that appeared normal and may or may not be infected with PRRSV, fetuses that had been dead for several days or more, and fetuses that were covered with a sticky mixture of meconium and amniotic fluids. Some of the meconium-stained fetuses were alive and others appeared to have recently died, some of which had blood mixed into the meconium/amniotic fluid paste covering the fetus. In some fetuses that had recently died and were mildly autolyzed, the amniotic fluid appeared very dark and thick as if it was contaminated by blood. The meconium staining was the most consistent gross abnormality observed in fetuses infected with virus. Many of the PRRSV-infected fetuses also had gross lesions associated with their

umbilical cord and less often internal organs. The gross lesions in the umbilical cords ranged from segmental hemorrhagic areas 1 to 2 cm in length to full length involvement of the cord which was grossly distended with frank hemorrhage. Occasionally, the cord did not contain hemorrhages but was edematous and 2 to 3 times its normal size. Edema around the kidney, spleen and within the mesentery of the spiral colon was frequently seen along with a clear effusion in the thoracic and abdominal cavities. In several instances the fetus was viremic and appeared normal, but its placenta had a brownish-tan discoloration and was detaching from the uterus. All live fetuses that had gross lesions in their umbilical cord were viremic. A necrotizing arteritis with periarterial hemorrhage was found in their umbilical cords which was the most consistent microscopic lesion in fetuses from which virus was isolated. Sows 1 through 7 had endometritis and myometritis of varying degrees suggesting PRRSV may induce these lesions; however, these lesions are likely not pathognomonic for a PRRSV infection.

Some of the fetal gross lesions reported in this study have been reported in previous studies in which sows were naturally or experimentally infected with PRRSV and euthanized between 107 and 112 DG. In one study, meconium staining was observed on some dead fetuses although no frank hemorrhage or umbilical cord lesions were noted (164). Moreover, that report also described the placenta separating from the uterus and having a brownish-green appearance, similar to what is occasionally observed in our studies. Clear effusions have been previously reported in thoracic or abdominal cavities of fetuses suspected or known to have been infected with PRRSV (35, 135). Microscopic lesions of the uterine/placental tissues compatible with the present study have been observed in 2 studies. In one, lesions were seen in the uterine and placental tissues of naturally and experimentally infected sows (164), and in the second study, only uterine lesions were noted in experimentally infected sows (24). Gross lesions of the umbilical cord compatible with this report (Fig 1) have been observed in concurrent studies in our laboratory in which gilts were exposed to the NADC-8 strain of PRRSV at or about 90 DG. (unpublished observations) The gilts were allowed to farrow naturally and a similar spectrum of near-term fetal death to normal appearing, congenitally infected pigs was observed. In contrast, no gross abnormalities were observed in the umbilical cords of the control litters, all of which were apparently free of PRRSV.

The significance of the diffuse lymphoplasmacytic placentitis with mild arteritis in the placenta associated with one fetus in litter 8 and 2 fetuses in litter 3 is unknown. Sow 8 had been previously exposed to the NADC-8 isolate of PRRSV 567 days before her second challenge with homologous virus at 90 DG reported in this study. Evidence for transplacental infection of fetuses in litter 8 was

not found suggesting the previous PRRSV exposure induced a protective immune response in sow 8. Previous work in our laboratory has demonstrated homologous PRRSV immunity can be induced in sows that will protect their fetuses from transplacental infection with PRRSV (88). However, it is not known if the homologous protection precludes any replication of PRRSV in the immune host. If the placental lesions observed in litters 3 and 8 are PRRSV related, then the incidence of PRRSV-induced placentitis in this model is very low.

Based on the gross observations and the presence of umbilical cord lesions reported in this study, we speculate that hypoxia may be a cause of death for some of the fetuses infected during late gestation with PRRSV. This may occur by the disruption of normal blood flow in the umbilical cord due to the necrosis of arterial walls and hemorrhage into the perivascular tissues followed by distension of the cord and occlusion of the umbilical vessels. Occasionally, the integrity of the cord is lost and hemorrhage occurs into the amniotic cavity and the fetus may exsanguinate. In addition, the lesions associated with the placenta and uterus may explain the onset of abortions that are seen following natural or experimental infection; e.g., the uterine lesions may be severe enough to disrupt the maternal/fetal barrier resulting in fetal death and abortion.

These putative mechanisms could explain the findings of others; i.e., the absence of gross or microscopic lesions in aborted or stillborn fetuses following experimental or natural infections of the dam since the virus may have little direct effect on the fetus and the cause of death may be largely due to umbilical cord or uterine lesions. In this study gross and microscopic lesions were only found in transplacentally infected fetuses and we believe the spectrum of live-normal to live-abnormal to dead fetuses reported here suggests these lesions are attributed to a PRRSV infection. However, at this point we do not know if the fetal and maternal lesions are due to a direct cytolytic viral infection, or if the lesions are the indirect result of a viral infection by some as of yet unidentified mechanism.

One of the litters examined in this study was infected with the Lelystad isolate of PRRSV, this suggests that although antigenic differences and pulmonary virulence differences may exist for strains of virus (61), PRRSV has a similar mechanism for transplacental infection and subsequent fetal death. Further studies, e.g., temporal immunohistochemistry and/or in situ hybridization studies, are needed to demonstrate the mechanism of the necrotizing arteritis and elucidate the apparent differences in susceptibility between umbilical arteries and veins.

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CHAPTER 7. HOMOLOGOUS CHALLENGE OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS IMMUNITY IN PREGNANT SWINE

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Abstract

The clinical consequences of single or multiple exposure of pregnant gilts to porcine reproductive and respiratory syndrome virus (PRRSV) at various stages of gestation were determined. Thirty-three pregnant gilts were allotted to 6 experimental groups (5 to 7 gilts/group). Gilts of groups 1 to 5 were exposed to strain NADC-8 of PRRSV at the following times: group 1, gestation day (GD) 1; group 2, GD's 1 and 90; group 3, GD 30; group 4, GD's 30 and 90; group 5, GD 90. Virus exposure was by either intrauterine (GD 1) or oronasal (GD's 30 and 90) inoculation. Gilts of group 6 were kept as nonexposed controls. Gilts were either necropsied on or about GD 111 (groups 1 to 5) or were allowed to farrow (group 6). The detection of PRRSV in serum of fetuses and piglets (within 12 hours of birth) was considered evidence of transplacental infection. Transplacental infection and virus-induced death were and were not, respectively, confirmed for groups 3, 4, and 5 and for groups 1, 2, and 6. Collectively the results indicated that intrauterine exposure to PRRSV at GD 1 was without clinical effect (groups 1 and 2) and provided protection against subsequent exposure to the same strain of virus at GD 90 (group 2). The highest incidence of transplacental infection and fetal death followed a single exposure to PRRSV at GD 90 (group 5).

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease of swine characterized by late-term, maternal reproductive failure and respiratory disease (80). The reproductive failure is recognized as an acute increase from normal production values in late-term abortions, stillborn pigs, mummified fetuses, early farrowings, and full-term, weak and unthrifty pigs. The onset of respiratory disease is concurrent with the reproductive disease and has a high morbidity for all ages

of swine with a characteristic "thumping" and increased mortality (80). Field reports and serologic surveys have supplied most of the data for the epidemiology of the disease; which, like most infectious diseases, has a broad spectrum of clinical manifestations based on age of the host, virulence of the strain, husbandry practices and management. Following the onset of a PRRS epizootic the reproductive parameters of a swine herd typically recover within a few months to pre-PRRS production values. However, herds may undergo a chronic loss of production in nursery and grower pigs suggesting an endemic form of the disease (162). Anecdotal reports and field studies have indicated the immune response to PRRS virus (PRRSV) may range from an immunosuppressive state of unknown duration in piglets to a protective response of at least a few months duration in sows (44, 162).

Experimental infection of sows with various strains of PRRSV during late gestation [gestation day (GD) 84 to 93] has resulted in reproductive failure that is similar to field observations (24, 107, 112, 135, 175). However, exposure of sows to one strain of PRRSV during early (GD 30) (107) and mid (GD 45 to 50) (22, 107) gestation did not result in late-term maternal reproductive failure that is commonly observed during PRRS epizootics. Results from studies involving intrauterine exposure of sows to PRRSV (90, 171) or PRRSV-contaminated semen (171, 172) at or near the time of breeding have been equivocal, which indicates the putative infertility described in field reports may be difficult to experimentally reproduce. Numerous controlled studies have been completed that have characterized the immune response of swine to a primary PRRSV exposure (127, 198, 201); however, few reports are available on the consequences of a secondary exposure to virus, i.e., challenge of natural immunity.

The design of this study was twofold; firstly, to test the pathogenicity of the NADC-8 strain of PRRSV at different stages of gestation, and secondly, to determine if the dam's immune response to virus exposure during early gestation would be protective against a subsequent homologous virus challenge during late gestation.

Materials and methods

Experimental design --Thirty-three pregnant gilts from a previous study (90) were allotted to 1 of 6 experimental groups in a modified-block design (Table 1) at GD 30. Pregnancy status was determined by failure to return to estrus following natural breeding and examination with a hand-held ultrasound device every other day beginning at GD 22. Pregnancy was confirmed at necropsy, by farrowing, or the discovery of aborted fetuses. Groups 1 and 2 had been previously

exposed to PRRSV at GD 1 by way of an intrauterine inoculation following natural breeding (90). All of these animals developed PRRSV-specific antibody within 2 weeks of inoculation and were clinically normal throughout their first 30 days of gestation. All gilts in groups 3 to 6 were free of PRRSV-specific antibody at GD 30. Groups 1, 3, and 5 were exposed to PRRSV at GD 1, 30, or 90, respectively. These groups were designed to serve as virus-exposed control groups. Groups 2 and 4 were exposed to virus at GD 1 and 30, respectively; these exposure times were used as an immunization against a subsequent homologous virus challenge at GD 90. Group 6 was the sham-infected control group. All virus exposure at GD 30 and 90 was via the oronasal route.

Table 1. Porcine reproductive and respiratory syndrome virus (PRRSV) exposure and necropsy schedule of pregnant swine.

Group	Day of Gestation			
	1	30	90	111
1 (n=5)	IU ^a		S ^b	Yes ^c
2 (n=5)	IU		ON ^d	Yes
3 (n=7)	S	ON		Yes
4 (n=5)	S	ON	ON	Yes
5 (n=6)	S		ON	Yes
6 (n=5)	S		S	Yes/No ^e

^aIU, Intrauterine exposure to PRRSV in previous study. ^bS - Sham inoculum or virus-free media.

^cYes, Gilt scheduled for necropsy on or about 111 days of gestation. ^dON, Oronasal exposure PRRSV in current study. ^eYes/No, 1 gilt was necropsied, the remaining 4 were allowed to farrow.

The groups were housed in separate outdoor facilities except for times following inoculation with PRRSV; at these times, gilts were housed in biocontainment facilities for 21 days-post-exposure (DPE). Blood samples were collected from gilts prior to virus exposure and at selected times thereafter. Gilts were either necropsied on or about GD 111 (groups 1 to 5) or allowed to farrow (group 6). Presence or absence of transplacental infection was determined by detecting virus in the serum of fetuses at necropsy or in the serum of piglets within 12 hours of birth.

Virus --Strain NADC-8 of PRRSV was used to infect gilts during this study. It had been isolated in our laboratory by exposing a culture of porcine alveolar macrophages to serum of a 2-day-old, moribund piglet from a naturally occurring case of PRRS in Iowa (all of the piglet's littermates had

either died *in utero* or were stillborn). A 4-week-old piglet was oronasally inoculated with 2 ml of medium collected from the macrophage culture. Serum was harvested from the piglet 7 days post-inoculation and was used to inoculate a PRRSV permissive cell line, MARC-145 (81). The virus was subsequently propagated (passaged) 3 times in cell culture and the medium harvested to produce a stock virus that was aliquoted into 5 ml volumes and stored at -80°C. The infectivity titer of the stock virus (i.e., the 3rd passage in MARC-145 cells) was $10^{5.7}$ CCID₅₀/ml. Stock virus was found free of contamination with several other porcine viruses known or suspected to be associated with porcine reproductive failure (encephalomyocarditis virus, porcine parvovirus, pseudorabies virus, and swine influenza virus) by administering an aliquot of the stock virus oronasally to two, 14-day-old, hysterectomy-deprived colostrum-deprived pigs and subsequently testing (4 weeks postexposure) their sera for antibodies to these viruses by neutralization or hemagglutination inhibition tests.

A sham inoculum was prepared from cell culture medium recovered from flasks of MARC-145 cells treated similarly as the virus infected flasks; the inoculum was aliquoted and stored at -80°C until use.

Necropsy procedures --Gilts were euthanized with an intravenous injection of pentobarbital and their uteri were recovered for examination of fetuses. Sections of the tonsil and uterine lymph node were collected from the gilt. Alveolar macrophages (AM) were collected at the time of necropsy as follows. The trachea and esophagus were severed caudal to the larynx and with blunt and sharp dissection the trachea, lungs, esophagus, and heart and great vessels were lifted free of the thoracic cavity. The dorsal surface of the lungs was rinsed free of blood and the aorta and esophagus were removed exposing the trachea at its bifurcation. The right bronchus was opened in an aseptic manner and 200 ml of lavage fluid [Eagles's minimal essential medium supplemented with gentamicin sulfate (0.5 mg/ml)] was dispensed into the right bronchus and lung; the lung was gently massaged to distribute lavage fluid throughout its lobes followed by aspiration of lavage fluid by pipette. Fetuses were removed from the uterus and their relative uterine position recorded, e.g., a fetus located next to the ovarian tip of the left uterine horn would be identified as L-1 and the next fetus as L-2, etc. Each fetus was categorized as live or dead based on detection of a heart beat or obvious postmortem autolysis or mummification. Crown-rump length (CR) was determined for an estimation of fetal age (100). Blood samples were collected from the base of the heart of live fetuses with a syringe and needle and thoracic fluids were collected from fetuses that had recently died in utero. All fluids and

tissues were stored at -80°C until tested for the presence of virus. Sections of lung from all fetuses that had died in utero were examined for porcine parvovirus antigen by immunofluorescence microscopy as previously described (105).

Virus Isolation --Serum, thoracic fluids, and tissues were tested for infectious virus as previously described (113) except for the volume; a 0.1 ml aliquot of fluid or a tissue suspension (10%) was tested for virus. Lavage fluid containing AM was tested for the presence of virus by cocultivation as previously described (113). Briefly, a 20 ml aliquot of lung lavage was absorbed for approximately 1 hour onto a 72-hour-old monolayer of MARC-145 cells in a 75 cm² flask. Medium was changed, removing nonadherent lung-lavage cells, and the monolayer observed daily for CPE. Medium was collected 4 and 5 days post inoculation and tested for virus by inoculating Leighton tubes containing glass coverslips with a MARC-cell monolayer. Approximately 24-hours-post inoculation the coverslips were fixed and reacted by indirect immunofluorescence microscopy with a PRRSV-specific, porcine hyperimmune serum and fluorescein isothiocyanate labeled goat anti-porcine immunoglobulin serum.

Serology --Serum was harvested from blood samples and stored at -80°C until tested for PRRSV-specific antibody by immunofluorescence microscopy as previously described (113).

Results

Determination of pregnancy --Thirty-one of thirty-three gilts deemed pregnant at GD 30 were confirmed pregnant at necropsy, by farrowing, or by abortion. Two gilts in group 4 thought to be pregnant may have aborted following virus exposure (details are presented in the following section).

Infection of the gilt --Clinical signs associated with primary PRRSV exposure were observed in 4 of 28 gilts; none of the 8 that received a secondary exposure to PRRSV developed clinical signs. The clinical signs observed ranged from a mild anorexia noted in 3 gilts to one gilt in group 4 that developed gross lesions typically described as "Blue Ear".

In group 1, one abortion occurred at GD 104, or 14 DPE of the sham inoculum. This animal was in an isolation room by itself from GD 90 to 102, at which time a second gilt from group 1 was moved into the isolation room and exposed to sham inoculum at GD 90. The animals were separated the following morning (GD 103) since they had been fighting during the night. The gilt aborted

during the night and a litter of 12 normal piglets was found the next morning (GD 104); they were nonviable, and virus was not detected in their sera. The other gilt appeared unaffected by the event and her litter was normal when examined at GD 111. In group 3, aborted fetuses were found in the pens of 2 gilts 6 and 10 days after their primary virus exposure at GD 30. In group 4, one gilt developed "Blue Ear"; this animal developed a deep, purplish discoloration of her ears, tail and lower extremities at GD 38, i.e., 8 DPE. A thick, whitish vulvar discharge was noticed at this time and she appeared listless and anorexic. The intensity of the cutaneous discoloration waxed and waned for the next 36 hours; during this time she had an elevated body temperature that peaked at 40.2°C. Within 3 days the gilt appeared normal, her temperature was 38°C, and she had regained her appetite. This gilt and a second gilt in group 4 are believed to have aborted following their GD 30 virus exposure; however, no evidence of abortion was observed in either pen. In group 5, two gilts aborted 14 and 17 DPE, respectively.

Regardless of exposure route, all gilts developed virus-specific antibody within 14 days of primary inoculation. Virus was isolated from serum of 8 gilts tested 7 DPE, one gilt at 7 and 14 DPE and one gilt at 14 DPE (Table 2). In gilts receiving a homologous exposure, PRRSV was not detected in any sera harvested at 7, 14, or at necropsy 21 DPE. At necropsy, virus was isolated only from gilts in group 5 that had been exposed to virus 21-days previously. Virus was isolated from alveolar macrophages collected from 6 of 6 gilts, lymph nodes from 3 of 6 gilts, and tonsils from 2 of 6 gilts.

Incidence of transplacental infection --Virus was not isolated from fetal or piglet serum in groups 1, 2, and 6 (Table 3) whereas it was isolated from groups 3, 4, and 5. In group 3, 1 and 3 fetuses were found in the respective pens of 2 gilts that aborted 6 and 10 DPE. The crown-rump lengths of the fetuses were compatible with their gestational age and no virus was recovered from their sera or associated remnants of fetal membranes. Virus was isolated from 1 fetus in 1 of the 5 litters examined at or about GD 111. In group 4, 2 gilts were thought to have aborted following their exposure to virus at GD 30; however, no fetuses or fetal membranes were found. Virus was detected in serum of 1 fetus in 1 of the 3 litters examined at or about GD 111. In group 5, exposure of gilts to PRRSV at or about GD 90 reproduced some of the clinical signs reported during field epizootics, i.e., late-term abortions and in utero fetal death. Two gilts aborted 14 and 17 DPE of the dam and virus was isolated only from the litter that aborted 17 DPE. Transplacental infection did occur in the remaining 4 litters that were examined 21 DPE.

Table 2. Results of virus isolation conducted on maternal serum and tissues following virus exposure and at necropsy.

Group	<u>Primary exposure</u>		<u>Secondary exposure</u>		<u>Necropsy</u>			
	7 DPE ^a	14 DPE	7 DPE	14 DPE	Serum	Alveolar Mφ	Uterine lymph node	Tonsil
1	3/5 ^b	0/5	0/5	0/5	0/5	0/5
2	2/5	0/0	0/5	0/5	0/5	0/5	0/5	0/5
3	1/7	2/2	0/5	0/5	0/5	0/5
4	0/5	0/0	0/3	0/3	0/3	0/3	0/3	0/3
5	3/6	0/6	0/6	6/6	3/6	2/6
6	0/5	0/5	0/1 ^c	0/1	0/1	0/1

^aDPE, Days post-exposure of PRRSV. ^bNumber of positive gilts/number of gilts in group. ^cFour of the five control gilts were allowed to farrow and were not necropsied. ... Not done.

Incidence of fetal death --The average number of dead, live, and live infected fetuses per litter was tabulated for each group (Table 3). No evidence for transplacental infection was found in groups 1, 2, and 6 which, collectively, had a ratio of dead: live of 7:131 or a 5% mortality rate. Based on CR lengths the estimated times of death for these fetuses were distributed throughout gestation; 4 fetuses died during the first half and 3 during the second half of gestation. Transplacental infection was detected in groups 3, 4, and 5 which had a mortality rate of 34%, 35%, and 25%, respectively. Collectively, these groups had a ratio of dead: live of 40:89 or a 31% mortality rate. In groups 3 and 4, 20 of the 25 dead fetuses were determined to have died during the second half of gestation and in group 5, all 15 fetuses died after GD 90.

In group 3, the single fetus (CR = 24 cm) that was viremic was located in one uterine horn that contained 6 dead fetuses (CR range 10 to 18 cm) while the contralateral uterine horn contained 5 live, normal fetuses (CR range 30 to 31.5 cm) (Fig. 1). In group 4, the single fetus (CR = 27.5 cm) that was viremic was the only live fetus found in the uterus. It was located at the ovarian tip of the right uterine horn and the remaining 9 dead fetuses (CR range 13.5 to 25.5 cm) were distributed throughout the uterus with the most recent deaths having occurred nearest the live, infected fetus (Fig. 2).

Serology --All gilts developed PRRSV-specific antibody within 14 DPE of the primary exposure and were positive throughout the study. No amnestic response was detected in gilts following homologous virus challenge (Table 4).

Table 3. Incidence of reproductive failure in gilts exposed to porcine reproductive and respiratory syndrome virus (PRRSV) and results of virus isolation conducted on fetal serum.

Group	Abortions ^a	Transplacental Infection	Live fetuses	Dead fetuses
1	1/5 (0) ^b	0/4 ^c	10.0 ^d (0) ^e	0.6 ^f
2	0/5	0/5	8.4 (0)	0.4
3	2/7 (0)	1/5	5.8 (.2)	3.0
4	2/5	1/3	6.0 (.3)	3.3
5	2/6 (1)	4/4	7.7 (2.7)	2.5
6	0/5	0/5	7.8 (0)	0.4

^aNumber of aborted litters that occurred in group/number of litters in group. Aborted litter in group 1 attributed to fighting. Two gilts in group 4 are suspected of having aborted; details presented in text.

^b(), number of aborted litters transplacentally infected with PRRSV. ^cNumber of litters with transplacental infection/number of litters in group. ^dAverage number of live fetuses per litter for group. ^e(), average number of live infected fetuses per litter for group. ^fAverage number of dead fetuses per litter for group.

Table 4. Indirect fluorescent antibody tests on serum from gilts exposed to PRRSV.

Group	0	14 ^a	21	30	60	81	90	110
1(n=5)	<4	10.4 ^b	...	10.8	8.4	7.2 ^c
2(n=5)	<4	8.8	...	9.5	8.0	7.6 ^c
3(n=5)	<4	9.2	8.0 ^c		
4(n=3)	<4	10.0	8.0	8.7 ^c		
5(n=6)	<4	11.0	11.6 ^c					
		9.9 ^d	11.6	10.2	8.0	8.4	8.2	7.4

^a Days post primary exposure. ^b Mean titer log2 for group. ^c Sample collected at necropsy

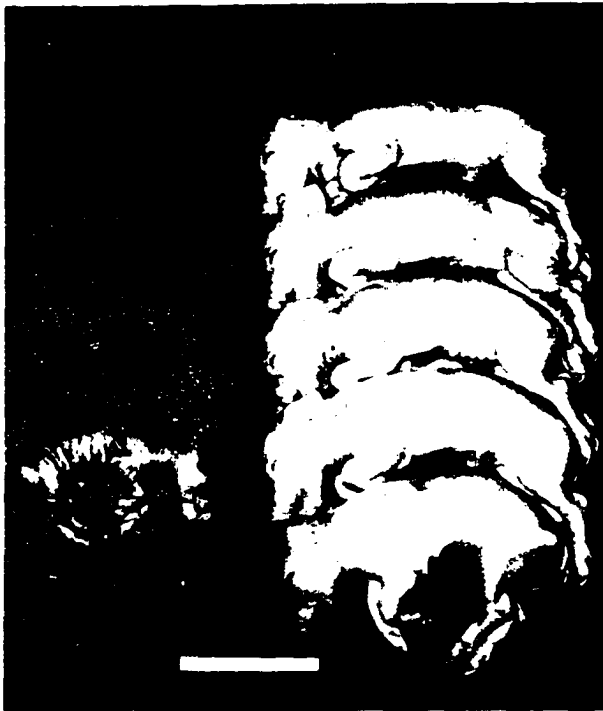
^d Mean titer for groups. ... Samples not collected.

Conclusion

This study has demonstrated that swine can be protected against a homologous PRRSV challenge as demonstrated in group 2, gilts exposed to virus at GD 1 and 90; no evidence of transplacental infection was found nor was any virus recovered from maternal serum or tissues at the time of necropsy. In contrast, group 5, gilts exposed to virus only at GD 90, had transplacental infection in 5 of 6 litters and virus was isolated from 6 of 6 gilts at necropsy. Transplacental

Fig. 1. Litter from gilt in group 3 exposed to PRRSV at 30 days of gestation and examined at 111 days of gestation. PRRSV and PRRSV-specific antibody was identified in the serum from the one live fetus in the left uterine horn. Neither virus nor virus-specific antibody were identified in the sera of the 5 live fetuses in the right uterine horn.

Fig. 2. Litter from gilt in group 4 exposed to PRRSV at 30 and at 90 days of gestation and examined at 111 days of gestation. PRRSV and PRRSV-specific antibody was identified in the serum from the one live fetus.



infection was not detected in one litter which aborted 14 DPE of the dam; however, virus was isolated from the uterine lymph node, tonsil, and AM of this gilt.

Isolating virus from alveolar macrophages cocultivated with a permissive cell line is a good method for evaluating the host's ability to clear a PRRSV infection. Previous work has demonstrated that detecting virus in alveolar macrophages may be the most sensitive test for detecting long-term virus replication in young pigs(113). The isolation of virus from alveolar macrophages, tonsil and lymph node tissues collected from group 5 and the absence of virus from groups 1 through 4 indicates gilts may clear a PRRSV infection by 80 DPE as found in group 3. The isolation of virus from alveolar macrophages collected 21 DPE in group 5 suggests this method also may be the most sensitive test for detecting long-term virus replication in the adult. In groups 2 and 4, gilts necropsied 21 DPE of homologous challenge, the absence of virus in alveolar macrophages and maternal tissues suggest the host did not replicate the virus as extensively as gilts in group 5 following a primary exposure. The lack of transplacental infection in group 2 and apparent absence of virus in the gilts at necropsy suggests they are capable of developing a protective immunity against a homologous virus challenge.

Experimental infection of sows with PRRSV between GD 77 and 93 has resulted in transplacental infection and production of the typical clinical signs observed in PRRS epizootics, i.e., late-term abortions and fetal death, early farrowings, stillborn and weak piglets (24, 107, 112, 135, 175). The NADC-8 isolate of PRRSV had similar pathogenic characteristics as demonstrated in group 5, gilts exposed to virus at or about GD 90. Moreover, this isolate may be more virulent during early gestation when compared to previous studies conducted with a different isolate administered to gilts between GD 30 and 70 (107) and to sows at GD 45 to 50 (22). In those studies, the typical reproductive failure that develops within several weeks following maternal exposure after GD 90 did not occur following exposure during early gestation; however, virus was recovered from some piglets at term. This apparent difference is not unprecedented since strains of PRRSV have been described which exhibit low or high pathogenicity for the respiratory (61) and reproductive tract (112). The mechanisms for the apparent difference in pulmonary and reproductive pathogenicity are unknown and may reflect one or more variables; e.g., dose, route, passage history, tissue tropism, etc. It is yet to be determined if any relationship exists between a strain's ability to cause respiratory disease and induce reproductive failure.

In a previous study (85), fetuses infected with PRRSV by direct, transuterine inoculation during early (GD 34 to 49), mid (GD 65), and late (GD 85) gestation, appeared to have a different

susceptibility to PRRSV based on gestational age, i.e., older fetuses would die sooner after direct inoculation with virus than younger fetuses, which would replicate the virus and appear normal for weeks. In the present study a significant amount of mortality occurred in groups 3 and 4, gilts exposed to virus at GD 30, when compared to groups 1, 2, and 6, litters that did not experience transplacental infection. The litters in groups 1 and 2 were normal in appearance and thought to be free of PRRSV, suggesting they were protected from homologous virus challenge and therefore similar to the control litters of group 6. Three of the 7 fetuses that were found dead in the litters of groups 1, 2, and 6 were estimated to have died during the second half of gestation. This is in contrast to groups 3 and 4, in which 80% (20 out of 25) of the mortality was estimated to have occurred during the second half of gestation (\geq GD 57 or CR length \geq 12 cm), with 15 of these fetuses dying between GD 57 and 77 based on CR length of mummified fetuses. The CR length of a mummified fetus probably underestimates its age due to the fact its soft tissues have shrunk and the measurement actually represents the skeleton of an older fetus. With this assumption, most of the fetal deaths may have occurred after GD 65 to 70; a time interval of at least 35 to 40 days after the dam was exposed to PRRSV.

The interval between exposing the dam during late-gestation to PRRSV and subsequent fetal infection and death can be estimated, based on data from this and the companion study (91). In the present study, one gilt in group 5 aborted 17 DPE and virus was recovered from her fetuses. In the companion study, a sow was oronasally exposed to virus at GD 90 and aborted 12 days later. Her litter contained transplacentally infected fetuses, thus marking, in our experience, the shortest confirmed interval between maternal exposure and fetal infection. This finding is consistent with our previous reports in which PRRSV-specific antibody was detected in serum from newborn piglets whose dams had been exposed to PRRSV 22 to 24 days prior to farrowing (107, 112). If the interval between infection and development of antibody is similar between young pigs and fetuses older than 90 days of gestation, then these newborn piglets would have been infected with virus for at least 10 to 14 days prior to birth. This observation suggests transplacental infection may have occurred 8 to 12 days after exposure of the dam, a time interval that is compatible with this and the companion study (91). Litters in group 5 contained dead fetuses that were judged to have died at least several days before necropsy of the dam at or about GD 111. Our interpretation of this data suggests the interval between exposing the dam to PRRSV late in gestation and the onset of fetal death is somewhere between 2 and 3 weeks. The evidence in groups 3 and 4 suggests a longer time interval exists between exposure of the dam and the onset of fetal death. A couple of possibilities may exist

for this increased interval; 1) fetuses are transplacentally infected within a couple of weeks and are able to replicate virus until they begin to die around GD 65 to 70, or 2) the dam is exposed to virus and harbors the virus for several weeks until transplacental infection occurs and the fetuses then succumb to virus infection within 2 to 3 weeks. Although previous direct, experimental infections of the fetus may support the first scenario, further studies are needed to test these hypotheses before any conclusions can be made.

An observation in this study that may help clarify cases from the field is the presence of 2 litters, each containing 1 infected fetus with littermates that have died and are mummified. Although we do not know the exact cause of death for these fetuses, we assume that an infectious agent played a role based on the progression of fetuses dying at different stages of gestation (Fig. 1 and 2). Based on our experiences, the possibility of isolating PRRSV from a fetus that has recently died is very low and isolating this virus from a mummified fetus is probably zero. We did rule out porcine parvovirus as a possible cause of death in this study based on fluorescent antibody tests conducted in our laboratory. Both of these litters and other litters in groups 3 and 4 suggest that PRRSV can establish a fetal infection following a single exposure of the dam at GD 30.

Group 4 was originally designed to test the possibility that a primary exposure at GD 30 would immunize the animal against a second exposure at GD 90. We believe all 5 gilts were pregnant at the time of the first exposure and the virus caused reproductive failure in at least one gilt (Fig. 2) and possibly in 2 additional gilts, both thought to have aborted following virus exposure at GD 30. We were unable to evaluate the effect of a second PRRSV exposure in this group because of these complications although we believe the second exposure at GD 90 had little effect on the gilts and their fetuses based on two litters which had 9 live and 1 dead and 8 live and 0 dead fetuses, respectively. Apparently, both of these litters were not infected following primary maternal exposure although both dams seroconverted to PRRSV and one was viremic at 14 DPE or GD 44. Both of these litters also escaped infection following secondary exposure of the dam at GD 90 which suggests these litters were protected against homologous challenge as in group 2.

A field report (73) of PRRS epizootics has described occasional abortions in swine during early gestation, which is compatible with observations in this study. Two of the gilts in group 3 were known to have aborted and 2 in group 4 were thought to have aborted; it is possible that these abortions are attributed to PRRSV, however, we were unable to identify virus in the fetuses or remnants of fetal membranes that were found. The absence of virus may be due to several factors;

PRRSV did not cause the abortions, the small amount of tissues available for testing, or the uterine lining becomes incompatible with pregnancy and fetal infection is not required for abortion.

The onset and duration of protective immunity in sows was unknown at the initiation of this study. The timing of the primary exposures was selected to allow enough time for protective immunity to develop before the subsequent homologous challenge at GD 90, a time when fetuses are very susceptible to transplacental infection. It appears that homologous protective immunity may develop within 60 DPE, as in the case of 2 gilts in group 4, and lasts at least through 90 DPE as found in group 2. The character of the proposed immunity described in this study is not known.

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CHAPTER 8. DURATION OF HOMOLOGOUS PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS IMMUNITY IN PREGNANT SWINE

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Abstract

The duration of porcine reproductive and respiratory syndrome virus (PRRSV) homologous immunity was tested in this study and found to last for at least 604 days post experimental exposure to field PRRSV. Eleven gilts (group A) received a primary exposure to field PRRSV by either an oronasal (n=6) or an intrauterine (n=5) route. The gilts were naturally bred at selected times (143 to 514 days) after primary virus exposure. They were oronasally exposed a second time to the same strain of virus on or about gestation day 90. Ten age-matched control sows free of PRRSV-specific antibody from the same source farm (group B) were naturally bred and were oronasally exposed to aliquots of the homologous challenge virus on or about gestation day 90. Nine of the 11 gilts in group A and all animals in group B became pregnant following one breeding cycle. The 2 nonpregnant gilts in group A were each naturally bred during 4 additional estrus cycles and neither one became pregnant. They were exposed to homologous challenge virus 562 and 604 days post primary exposure, respectively. All animals were necropsied 21 days post homologous challenge. Sera and alveolar macrophages from each dam and sera from each fetus were tested for virus. Transplacental infection was detected in 0/9 and 8/10 litters in groups A and B, respectively. Virus was detected in 0/11 and 10/10 of the alveolar macrophage samples collected in groups A and B, respectively. Serum was harvested at selected times throughout the experiment and tested for PRRSV-specific antibody by indirect immunofluorescence microscopy. All gilts in group A were seropositive for the duration of the experiment and all animals in group B seroconverted following exposure to field PRRSV. This study demonstrates adult swine can produce a homologous protective immunity following PRRSV exposure which may persist for the production life of the animal.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a disease of swine first observed in the United States during the late 1980's as epizootics of maternal reproductive failure and respiratory disease (80). The disease soon spread throughout North America and was reported in Europe by the fall of 1990 (189). Although the etiologic agent was unknown in the beginning, the disease was thought to be infectious and caused by a virus. This was confirmed by Dutch scientists who isolated a virus and demonstrated it as the causative agent of PRRS in 1991 at the Central Veterinary Institute in Lelystad, The Netherlands (175). The virus is classified as a member of the proposed virus family, Arteriviridae, and it is known as the PRRS virus (PRRSV) (117). Since its discovery, numerous studies have been published describing the molecular biology of the virus and the epizootiology and pathogenesis of the reproductive and respiratory disease. However, limited information is available that characterizes the host's immune response to a primary PRRSV exposure and the formation and duration of the specific immune response.

Field reports suggest sows develop a protective immunity following PRRSV-induced reproductive losses based on observations that affected sows have a normal litter following rebreeding despite the apparent circulation of virus within the breeding herd (2, 13, 39, 162). The duration of this proposed protective immunity is unknown; however, the duration of detectable PRRSV-specific antibodies that develop in sows following natural infection is thought to be short-lived, i.e., 4 to 8 months (2, 38, 41). Experimental studies have demonstrated in swine of all ages that PRRSV-specific antibody persists for the duration of the experiment; however, most animal studies have been designed as short-term experiments which usually conclude within several weeks of primary virus exposure. Limited studies have been completed characterizing the long-term kinetics of the PRRSV-immune response in which specific antibody was shown to persist for 105 (108, 201), 220 (195) and 340 (127) days in experimentally infected pigs.

In the study reported previously (92), we demonstrated homologous PRRSV-protective immunity was experimentally produced within 90 days-post-exposure (DPE) and virus-specific antibody was detected for at least 110 DPE in adult swine. The protective immunity was based on two criteria; 1) the absence of transplacental transfer of challenge virus and 2) the apparent lack of virus replication in the dam 21 DPE to challenge virus. The purpose of the study reported here was to test the duration of a protective homologous immune response following challenge of swine with field-type PRRSV.

Materials and Methods

Experimental design --Group A consisted of 11 gilts (nos 1-11) which had previously been exposed one time to PRRSV either by way of an intrauterine route at gestation day (GD) = 1 (90) or an oronasal route at GD = 30 (92) (Table 1). All of these gilts failed to farrow; 6 failed to conceive, 2 were thought to have aborted and 3 were known to have aborted. All 11 animals were oronasally exposed a second time to PRRSV between 233 and 604 days post primary exposure. Group B, consisted of 10 age-matched sows (nos 12-21) free of PRRSV-specific antibody that were either control animals from a previous study (90) (n=4) or purchased from the original source herd (n=6). For the purposes of this paper all animals in each group will be referred to as sows. Each group was housed separately in outdoor facilities and was naturally bred at selected times. The experimental model for challenge of protective immunity consisted of an oronasal exposure of all sows of groups A and B to live virus on or about GD 90. Twenty-one days following virus exposure all sows were euthanized and maternal and fetal tissues were tested for infectious virus. Blood samples were collected from sows in group A at various times following primary exposure to virulent virus. Following homologous challenge in groups A and B, blood samples were collected at 0, 7, and 21 DPE. Two factors were used to evaluate the duration of protective immunity in group A when compared to group B: 1) the presence of PRRSV in alveolar macrophages (AM) of the dam, and 2) the presence of transplacental infection. Procedures for necropsy, virus isolation and serology were performed as previously described (92).

Virus --Animals were exposed to one of two viral inoculums prepared in a similar fashion from the NADC-8 isolate of PRRSV (90). Viral inoculum #1 was the original virus used for primary challenge of group A. Approximately 1 year after the virus had been propagated and stored at -80°C, sows 1-6, and 12-15 were challenged with aliquots of the this stock virus. Due to the duration of this experiment and the diminishing stock of original virus, a second stock of virus was prepared following the same protocol as previously described (90). Aliquots of this second viral inoculum were administered to sows 7-11, and 16-19 within 8 weeks of storage. Two aliquots of the original virus stock, viral inoculum #1, were tested for pathogenicity approximately 2 years after its production by experimental inoculation of control sows 20 and 21.

Results

Infection of the dam --None of the sows in groups A or B had displayed any clinical signs following exposure to PRRSV except for spontaneous abortions in sows 13 and 16; 21 and 12 DPE, respectively. Virus was not isolated from any serum or AM sample collected from any sow in group A. In contrast, virus was isolated from serum of 8 of 10 sows in group B tested 7 DPE and virus was isolated from the AM of 10 of 10 sows tested 21 days post exposure (DPE) (Table 1).

Incidence of transplacental infection --No virus was detected in fetal sera from any litter in group A. Virus was detected in at least 1 fetus from 8 of the 10 litters in group B (Table 1). Transplacental infection was not detected in the remaining 2 litters in group B; one of which consisted of 2 live fetuses and the second consisting of 5 live fetuses and 2 small mummies (Crown Rump length = 4.5 and 5.0 cm).

Incidence of fetal death --The overall mortality rate for fetuses in group A was 2% (2 dead: 112 live) vs. group B which was 33% (38 dead: 77 live). The 2 dead fetuses in group A and 6 of the dead fetuses in group B were estimated to have died prior to 90 days of gestation, the time of virus challenge for the sows. If the mummified fetuses are removed from the calculation of mortality rates, then the incidence of fetal death following challenge of the sows was 0% vs. 29%, for Group A vs. Group B. The average number of live, infected fetuses per litter in group B was 5.

Serology --Throughout the duration of this study PRRSV-specific antibody was detectable by IFA in all sows of group A (data not shown). No detectable anamnestic response was detected in the sows of group A following homologous virus challenge (Table 1). All sows in group B developed PRRSV-specific antibodies following challenge (Table 1). Six of the 8 transplacentally infected litters in group B had one or more live fetuses with detectable PRRSV-specific antibody (Table 1).

Discussion

This study has demonstrated that swine can be protected against a homologous PRRSV challenge for at least 604 DPE, which in modern production facilities is essentially a life-long protection. The first inoculum was about 1 year old when administered to sows 1 through 6 in group A and sows 12 through 15 in group B. Transplacental infection was not found 21 DPE in two (sows 14 and 15) of the four control sows in group B suggesting attenuation of stock virus may have occurred during its

Table 1. Results of virus isolation and serology conducted on sow and fetal tissues.

Group A Sow#	Primary exposure	Challenge ^a	Necropsy ^b	Sow				Fetal					
				Virus Isolation ^c				Serology ^d		Virus Isolation ^e			Serology ^f
				D0	D7	D21	Mφ	Challenge	Necropsy	Live	Dead	Mummy	Live
1	ON	233	254	-	-	-	-	256	256	12(0)	0	0	0
2	ON	273	291	-	-	-	-	256	256	14(0)	0	0	0
3	ON	295	316	-	-	-	-	256	256	13(0)	0	0	0
4	ON	305	326	-	-	-	-	256	64	9(0)	0	0	0
5	IU	320	341	-	-	-	-	1024	1024	12(0)	0	0	0
6	ON	349	370	-	-	-	-	256	256	15(0)	0	0	0
7	ON	479	500	-	-	-	-	256	256	17(0)	0	0	0
8	IU	554	575	-	-	-	-	256	256	5(0)	0	0	0
9	IU	562	583	-	-	-	-	1024	256	NP	NP	NP	NP
10	IU	567	588	-	-	-	-	1024	1024	15(0)	0	0	0
11	IU	604	625	-	-	-	-	256	256	NP	NP	NP	NP
Group B													
12	ON	0	21	-	+	-	+	<4	1024	5(3)	2(0)	0	5(1)
13	ON	0	21	-	-	-	+	<4	4096	8(6)	3(0)	0	8(4)
14	ON	0	21	-	+	-	+	<4	1024	5(0)	0	2(0)	5(0)
15	ON	0	21	-	+	-	+	<4	4096	2(0)	0	0	2(0)
16 ^g	ON	0	12	-	+	-	+	<4	256	14(9)	5(0)	2(0)	14(0)
17	ON	0	21	-	-	-	+	<4	1024	10(6)	2(0)	2(0)	10(0)
18	ON	0	21	-	+	-	+	<4	4096	10(8)	1(0)	0	10(7)
19	ON	0	21	-	+	-	+	<4	4096	11(11)	3(0)	0	11(9)
20	ON	0	21	-	+	-	+	<4	1024	4(4)	9(1)	0	4(2)
21	ON	0	21	-	+	-	+	<4	1024	8(5)	7(2)	0	8(4)

ON = Oronasal route of exposure. IU=Intrauterine route of exposure. Days post primary exposure to PRRSV when sow challenged with virus. Days post primary exposure when sow was necropsied. Virus isolation conducted on sera collected from sows at 0, 7, and 21 days post challenge and alveolar macrophages (Mφ) at the time of necropsy. Results recorded as positive (+) or negative (-). IFA titers on sow sera collected at time of challenge and at necropsy. Virus isolation on fetuses recorded as number of fetuses in classification (number of fetuses positive for virus). Number of live fetuses in litter (number of fetuses positive for PRRSV-specific antibody). NP= Not pregnant. Sow aborted 12 DPE.

storage at - 80°C. However, both of these sows seroconverted to PRRSV and the virus was recovered from their alveolar macrophages at necropsy; results which are consistent with the other sows in group B. Two aliquots of the first inoculum were oronasally administered to sows 20 and 21 approximately 2 years after its production; both of these litters experienced transplacental infection and fetal mortality which suggests the original inoculum or at least some aliquots of it did not lose pathogenicity due to storage and the lack of transplacental transfer of virus in sows 14 and 15 may have been due to chance. Of course, other possibilities may exist, e.g., some of the fetuses were infected and we were unable to isolate virus, or the maternal infection, due to some unknown reasons, was inadequate to cause a transplacental infection.

Animals 9 and 11 are referred to as sows although both were considered infertile; both were naturally bred 4 times each during the course of this study and neither conceived in contrast to the other 19 sows all of which conceived after one breeding to the same boar. The infertile animals are included in this study because they were part of the original experiment, have a similar virus exposure history, and were used to evaluate the dam's protection against homologous challenge by evaluating serum antibody and alveolar macrophages.

Isolating virus from alveolar macrophages cocultivated with MARC-145 cells is a good method of evaluating the host's ability to clear a PRRSV infection. Previous work has demonstrated that detecting virus in alveolar macrophages may be the most sensitive test for detecting long-term virus replication in the pig (108) and sow (92, 112). In this study PRRSV was recovered from the AM of all 10 sows tested in group B, which is identical to the results of the previous study in which virus was recovered from all 6 gilts exposed to PRRSV 21 days previously (92). The absence of virus in alveolar macrophages following homologous virus challenge in the 11 sows of this study and 8 sows from the previous study (92), suggests an immune host may not replicate PRRSV as extensively following homologous challenge as it did following the primary virus exposure.

In this study all sows in groups A and B developed virus-specific antibody following primary exposure to PRRSV and all sows in group A were positive by the IFA test for the duration of the experiment. No anamnestic response was detected in the sows in group A following homologous challenge.

Sow 16 aborted 12 DPE and virus was recovered from 9 of 14 live fetuses, thus marking in our experience the shortest time interval from oronasal infection of the dam to transplacental infection of her fetuses. Six of the remaining 7 transplacentally infected litters in group B contained one or more live fetuses with PRRSV-specific antibody. Assuming late-gestation porcine fetuses are capable of

developing an immune response to PRRSV in a similar time span as neonatal piglets, e.g., 10-14 days, we would then expect these seropositive fetuses to have been transplacentally infected at least 7-11 days after oronasal inoculation of the dam. This data is consistent with previous observations on the temporal development of antibodies in fetuses transplacentally infected with PRRSV (107).

This study indicates homologous protection can be experimentally induced and it has a long duration. Additional studies are necessary to evaluate the extent of heterologous protection following immunization of sows with field-type virus and to test the efficacy and duration of immunity in sows following the administration of commercially available PRRSV vaccines.

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CHAPTER 9. EVALUATION OF HOMOLOGOUS AND HETEROLOGOUS PROTECTIVE IMMUNITY IN GILTS WITH ANTIGENICALLY DISTINCT PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ISOLATES

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Abstract

Objective-To induce and evaluate homologous and heterologous porcine reproductive and respiratory syndrome virus (PRRSV) protective immunity in pregnant gilts.

Animals-40 pregnant gilts.

Procedure-Gilts were immunized by intrauterine administration at or before breeding of a field isolate of PRRSV from North America. Protective homologous and heterologous immunity were evaluated by oronasal exposure of these gilts during late gestation to either the same field isolate of PRRSV (homologous) or an antigenically distinct field isolate of PRRSV from Europe (heterologous). Gilt and fetal tissues were tested for the presence of challenge virus. Lack of challenge virus in gilt tissues indicated protective immunity. Lack of challenge virus in fetal tissues indicated protection from reproductive losses. Lack of challenge virus in gilt and fetal tissues indicated complete protection.

Results-Homologous protective immunity was complete. Heterologous protective immunity was incomplete; challenge virus was detected in the heterologous challenge group in the tissues of 5 of the 8 gilts and in fetal tissues in 1 of the 7 litters.

Conclusion-Homologous protective immunity can be induced in gilts by exposure to live PRRSV. Heterologous protection from reproductive losses can be induced in gilts by exposure to live PRRSV; however, this immunity may have a shorter duration than homologous immunity.

Clinical Relevance—This study suggests the use of live virus vaccines may provide some protection against PRRSV-induced reproductive losses in the field. However, the protection may have a limited duration and it may be dependent on the antigenic relatedness of vaccine and field virus.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first recognized in the United States during the late 1980's as epizootics of maternal reproductive failure and respiratory disease (80). Initially, the etiology was unknown although it was thought to be an infectious agent which was later confirmed by the discovery of the PRRS virus (PRRSV) by scientists at the Central Veterinary Institute in Lelystad, The Netherlands (189). Soon after the initial outbreaks, anecdotal reports described sows recovering from the disease and producing a normal litter in the subsequent gestation despite ongoing reproductive losses in the sow herd and respiratory disease in the nursery. These observations suggested that swine could develop protection from reproductive losses. Moreover, farms could follow certain protocols which might result in the elimination of the clinical disease, and possibly the virus from the herd (39, 40). These protocols were developed on the premise of the development of protective immunity.

We developed a model for demonstrating the development of homologous PRRSV protective immunity in swine by intrauterine inoculation of gilts and sows prior to or at the time of conception with live field PRRSV (NADC-8 virus) followed by an oronasal challenge with the same virus during late gestation when pregnant swine are the most susceptible to transplacental infection (107). In this model, a lack of detectable challenge virus in the immunized gilts and sows is considered proof of protective immunity. In addition to the protective immunity in the dam, transplacental PRRSV infection and reproductive losses did not occur. We have shown that gilts can develop homologous protective immunity and protection from reproductive losses within 90 days of immunization (92) and this immunity can last for at least 600 days (91). Complete homologous protection against reproductive losses has been demonstrated in gilts following immunization with either of two attenuated-virus vaccines (hereafter referred to as vaccines) which were derived from field virus isolated from the North American continent. In these studies, the interval of time between immunization and challenge was about 120 -140 days (54, 66).

Heterologous protection against reproductive losses in pregnant gilts following immunization with these vaccines has been reported. In one study heterology between the immunizing virus (vaccine) and the North American challenge virus (field) was defined by a virus neutralization test (68). In a second study, heterology between the immunizing virus (vaccine) and the European

challenge virus (field) was defined by published genetic differences and by an immunofluorescence microscopy (IFA) test (54). In both of these studies the heterologous protection was not complete in that transplacental infection occurred in some vaccinated gilts following field virus challenge. These results are in contrast to the previous homologous challenge studies in which no transplacental infection was found in the immunized gilts following challenge.

The duration and magnitude of the humoral immune response in swine following experimental infection (91, 127) with PRRSV appears greater than that following natural infection (2, 163) or vaccination.^a This apparent difference between laboratory and field results could be attributed to many factors, some of which may involve the antibody detection test and technical skill. However, in our experience utilizing similar conditions, we found a difference between the humoral immune response in gilts following experimental infection with virulent strains of PRRSV vs. experimental infection with an attenuated vaccine strain of PRRSV.^a We speculate this difference could be due to the quantity of live virus and/or the virulence of live virus used for immunizing the gilt. This raises the question of whether field virus immunization may induce a superior immune response that could be demonstrated with a heterologous virus challenge that is antigenically distinct from the NADC-8 virus. The objectives of the study reported here were to compare homologous and heterologous protective immunity in pregnant swine utilizing our model of field virus immunization followed by field virus challenge.

Materials and Methods

Experimental Design --This study consisted of two experiments. The objective of experiment 1 was to immunize gilts for use in experiment 2 by intrauterine administration of PRRSV at or near the time of conception (90). The objective of experiment 2 was to evaluate protective immunity during late gestation in immunized gilts after a homologous or heterologous oronasal viral challenge.

Animals --Forty gilts were purchased from a farm free of PRRSV-specific antibody and clinical signs of maternal reproductive failure. When the gilts weighed approximately 105 kg, 5 were transported at weekly intervals in to our laboratory. Upon arrival and again 3 weeks later, each gilt received an intramuscular (IM) injection of a multivalent inactivated commercial vaccine^b containing antigens for porcine parvovirus, *Erysipelothrix rhusiopathiae*, and *Leptospira* sp. One boar (approximately 110 kg) was purchased from the same farm and vaccinated accordingly. All groups of animals were

housed in open front barns until they were moved to indoor isolation facilities at the time of PRRSV exposure or at or about gestation day (GD) 90 for non-virus exposed controls.

Experiment 1 --Three weeks after arrival, a synchronization of estrus protocol (171) was initiated at weekly intervals for each group of 5 gilts. It consisted of altrenogest^c given orally at a dose of 13 mg per gilt every day for 13 days. Dinoprost trometamine^d was given IM at a dose of 10 mg per gilt on the morning of the 14th day and repeated 8 hours later. On day 15, a single IM dose per gilt was given of a product^e containing 300 IU human chorionic gonadotropin (HCG) and 600 IU pregnant mare serum gonadotropin (PMSG). Gilts were to be bred on the second and third day of estrus. Following the second natural breeding (or refusal of the boar), two of the five gilts in each group (total of 16 gilts) were moved to isolation facilities and each received an intrauterine administration of PRRSV (5×10^6 TCID₅₀) as previously described (90). The remaining 3 gilts in each group (total of 24) received a sham inoculum in a similar fashion. Blood was collected upon arrival to our laboratory, at the time of breeding/inoculation, and at selected times thereafter.

A difficulty arose in this experimental design where the boar expressed no interest in breeding any of the gilts. Semen was purchased from a boar stud that maintained boars free of PRRSV-specific antibodies. On day 18 and 19 of the estrus synchronization protocol (72 and 96 hours post administration of HCG and PMSG) the gilts were artificially inseminated one time each day. The virus or sham inoculum was mixed with the first semen dose and administered via an insemination pipette at the time of breeding = day 0. Following the artificial insemination of the last group of 5 gilts a second boar was purchased from the same source farm and used for estrus detection and natural breeding. Gilts that did not conceive after artificial insemination in the sham-inoculated group were bred as they came into estrus and then monitored daily for the next 28 days for a return to estrus. After the last gilt was bred in the sham-inoculated group, the boar was used to breed gilts in the virus-inoculated group that had not conceived. Pregnancy status was determined by examination of each gilt's uterus at the time of necropsy or by farrowing.

Experiment 2 --At or about GD 90 the gilts were blocked by virus or sham exposure and randomly allotted to one of five challenge groups with 8 gilts each (Table 1). Group A, the homologous virus-exposed controls, received a sham inoculum at GD 0 and homologous virus at GD 90. Group B, the homologous protective immunity group, received homologous virus at GD 0 and 90. Group C, the heterologous virus-exposed controls, received a sham inoculum at GD 0 and heterologous virus at

GD 90. Group D, the heterologous protective immunity group, received homologous virus at GD 0 and heterologous virus at GD 90. Group E, the non-virus exposed controls, received a sham inoculum at GD 0 and 90. All gilts in groups A, B, C, and D were euthanatized 21-days-post challenge (GD 111) and their uteruses removed. The serum from each fetus was tested for PRRSV. Transplacental infection for each litter was defined as the isolation of virus from the serum of a least one fetus in a litter. The lack of transplacental infection following challenge virus exposure of the dam was used as an indication of protection from PRRSV-induced reproductive losses. A lung lavage was collected from each gilt at necropsy and tested for virus. Protective immunity for each gilt was defined as the lack of detectable virus in the lung lavage of the gilt at the time of necropsy. Complete protection for a gilt was defined as a lack of detectable virus in her fetuses and lung lavage. In group E, all 8 gilts were allowed to farrow and blood was collected from the gilt and her piglets within 12 hours of birth. All group E gilts and pigs were transferred to different studies and monitored periodically for the development of PRRSV-specific antibody. For this study, abortions were defined as expulsion of fetuses before GD 112.

Table 1. Experimental design.

Group		Immunizing exposure ^a	Challenge exposure ^b
A	Challenge control-homologous virus	Sham	NADC-8
B	Protective immunity-homologous virus	NADC-8	NADC-8
C	Challenge control-Heterologous virus	Sham	Lelystad virus
D	Protective immunity- Heterologous virus	NADC-8	Lelystad virus
E	Control	Sham	Sham

^aImmunizing exposure = Intrauterine inoculation of either PRRSV (NADC-8 virus) or Sham inoculum. ^bChallenge exposure = Oronasal inoculation of either PRRSV (NADC-8 virus or Lelystad virus) or Sham inoculum.

Virus --A North American PRRSV isolate (NADC-8), was used for the immunization (intrauterine administration) of the 16 gilts at the time of artificial insemination and as the homologous challenge virus (oronasal administration) in groups A and B. A European PRRSV isolate (Lelystad virus), was used for the heterologous challenge virus (oronasal administration) in groups C and D. The NADC-8 virus was isolated from serum of a neonatal pig collected from a PRRS epizootic (90). The Lelystad virus (9th passage) was provided to us by Dr. Fry at the National Veterinary Services Laboratory who received the virus as a gift from the Central Veterinary Institute in Lelystad, The Netherlands.

Both virus stocks were prepared as follows. An aliquot of virus was oronasally administered to a naive sow at GD 90. The sow was euthanatized 21-days later and her fetuses examined for the presence of virus. PRRSV was isolated from the serum of one selected fetus and propagated 3 times on MARC-145 cells (81). The final virus titer was adjusted to approximately 10^6 TCID₅₀ of virus per ml, aliquoted into 5 ml doses and stored at -80C until use. A sham inoculum of cell culture was prepared in a similar fashion, aliquoted and stored at - 80C until use.

Virus Isolation and Serology tests --In our experiences, fetal serum has been the most sensitive tissue to test for PRRSV as an indication of transplacental infection. Serum or thoracic fluids were tested for virus in fetuses that had recently died, and thoracic tissues were tested in fetuses that were undergoing extensive postmortem autolysis and mummification. In the sow, the lung lavage has been the most sensitive tissue to test for PRRSV. Virus isolation was conducted as previously described utilizing 100 ul of fetal sera and 10 ml of sow lung lavage (92). Gilt sera were tested for virus-specific antibody by IFA tests as previously described (106) utilizing either the NADC-8 or Lelystad virus as antigen in the cell culture.

Statistics --Fisher's exact test (159) was used to determine significant differences in conception rates between infected and non-infected gilts, for significant differences in the incidence of protective immunity, and for transplacental infection. Results were considered significant if $P \leq 0.05$.

Results

Experiment 1: Clinical signs of infection --All 16 gilts in the intrauterine PRRSV-exposed group developed virus-specific antibody by day 14 (IFA titers ranged from 64 to 4096). Three of the 16 gilts had a 24 to 48 hour anorexia beginning 3 to 4 days post-infection. None of the 24 sham-exposed gilts developed PRRSV-specific antibody or anorexia between GD 0 and 90.

Conception rate --Following artificial insemination the conception rates for the intrauterine virus-exposed and sham-exposed groups were similar ($p = 0.52$), i.e., 4 of 16 gilts (25%) vs. 5 of 24 gilts (21%), respectively. Following natural service during one estrus cycle, the conception rates were similar between the virus-exposed and sham-exposed groups ($p = 0.26$), i.e., 8 of 10 gilts (80%) vs. 18 of 19 gilts (95%), respectively. All 3 of the nonpregnant gilts conceived following a second

Table 2-Results of indirect fluorescent antibody (IFA) and virus isolation tests for groups A, B, C, and D.

Group-Gilt	Interval ^c	IFA		Gilt ^a		Virus Isolation			
		Challenge ^d	Necropsy	Lung Lavage	T P	Fetuses ^b			
						Live	Dead	Mummy	
A-1	-	<4 (<4)	1024 (<4)	Yes	Yes	11(8)	5(1)	2(0)	
A-2	-	<4 (<4)	64 (<4)	Yes	Yes	8(8)	3(3)	3(0)	
A-3	-	<4 (<4)	256 (<4)	Yes	Yes	6(3)	2(0)	0	
A-4	-	<4 (<4)	64 (<4)	Yes	Yes	8(7)	4(0)	0	
A-5	-	<4 (<4)	256 (<4)	Yes	Yes	11(9)	3(1)	0	
A-6	-	<4 (<4)	64 (<4)	Yes	Yes	11(3)	0	1(0)	
A-7	-	<4 (<4)	256 (<4)	Yes	Yes	10(9)	3(0)	0	
A-8	-	<4 (<4)	64 (<4)	Yes	Yes	8(4)	1(1)	1(0)	
B-1	90	64 (<4)	64 (<4)	No	No	11(0)	0	1(0)	
B-2	90	64 (<4)	64 (<4)	No	No	8(0)	0	0	
B-3	91	64 (<4)	256 (<4)	No	N PG	
B-4	125	64 (<4)	64 (<4)	No	No	12(0)	0	0	
B-5	143	64 (<4)	64 (64)	No	No	13(0)	0	0	
B-6	155	4 (<4)	64 (<4)	No	No	8(0)	0	0	
B-7	167	256 (<4)	256 (<4)	No	No	9(0)	0	1(0)	
B-8	205	16 (<4)	64 (<4)	No	No	12(0)	0	0	
C-1	-	<4 (<4)	<4 (1024)	Yes	Yes	12(7)	2(0)	1(0)	
C-2	-	<4 (<4)	<4 (256)	Yes	Yes	11(4)	0	0	
C-3	-	<4 (<4)	16 (1024)	Yes	No	5(0)	0	2(0)	
C-4	-	<4 (<4)	4 (1024)	Yes	Yes	6(1)	0	0	
C-5	-	<4 (<4)	4 (1024)	Yes	Yes	14(5)	0	0	
C-6	-	<4 (<4)	<4 (4096)	Yes	Yes	15(7)	1(0)	0	
C-7	-	<4 (<4)	<4 (4096)	Yes	Yes	9(2)	1(0)	0	
C-8	-	<4 (<4)	16 (256)	Yes	Yes	15(14)	0	0	
D-1	90	64 (<4)	64 (4)	No	No	7(0)	0	4(0)	
D-2	91	64 (<4)	64 (16)	No	No	4(0)	0	0	
D-3	91	256 (<4)	1024 (256)	No	N PG	
D-4	134	64 (<4)	1024 (4)	Yes	No	11(0)	0	1(0)	
D-5	142	256 (<4)	64 (64)	Yes	No	14(0)	0	0	
D-6	156	64 (<4)	256 (16)	Yes	No	12(0)	0	0	
D-7	168	64 (<4)	64 (16)	Yes	No	11(0)	0	0	
D-8	170	256 (<4)	4096 (64)	Yes	Yes	13(5)	0	1(0)	

^aIsolation of virus from lung lavage of gilt, and presence of transplacental infection (TP). ^bCondition of fetuses at time of necropsy of gilt; live, dead = based on appearance fetus judged to have died in last few days, mummy = based on appearance fetus judged to have died more than one week prior to necropsy of gilt. Fetal virus isolation results reported as number of fetuses in category(number of fetuses positive for virus). ^cInterval = time in days between immunizing exposure and challenge exposure. - = No immunizing exposure, challenge exposure only. IFA antibody titer reported in 4-fold dilutions: Value without parenthesis = IFA test conducted with NADC-8 virus antigen; Value within parenthesis = IFA test conducted with Lelystad virus. ^dAll gilts challenged at or about gestation day 90 and necropsied 21 days later. NPG = not pregnant. Data for Group E, non-infected controls, not included in table. All animals in group E were free of virus and virus-specific antibody for the duration of the experiment.

natural breeding. Two of the nonpregnant gilts in the virus-exposed group were never bred by the boar.

Experiment 2: Clinical signs of infection –Except for two abortions, no clinical signs of disease were noted in any of the gilts following challenge at GD 90 with PRRSV. Gilt A-8 aborted 20 days after challenge (GD 111); the litter consisted of 8 live-born pigs, 1 stillborn pig and 1 dead fetus estimated to have been dead at least several days. Four of the live-born pigs were weak but had managed to suckle, of the remaining 4 live-born pigs, 3 had died soon after birth, and one was very weak and had not moved from behind the gilt. PRRSV was isolated from 4 of the 9 live-born pigs and from the stillborn pig. Two live-born pigs and the stillborn pig had gross and microscopic evidence of an umbilical cord vasculitis. Interstitial pneumonia and a mild hepatitis was observed in 5 and 2 of the 8 live-born fetuses, respectively. Gilt B-6 aborted 18 days after challenge (GD 107); the litter consisted of 8 stillborn pigs. PRRSV was not isolated from any of the stillborn pigs and they were normal in appearance.

Virus Isolation -- At necropsy, 21 days after challenge exposure, PRRSV was isolated from the lung lavage of 100%, 0%, 100%, and 63% of the gilts in groups A, B, C, and D, respectively (Table 2). Homologous protective immunity was demonstrated by a significant difference in the incidence of virus isolated from lung lavage between groups A and B ($p = 0.0001$). Heterologous protective immunity was not found in group D; i.e., no difference in the incidence of virus isolated from lung lavage between groups C and D ($p = 0.2$). Transplacental infection was identified in 100%, 0%, 88%, and 14% of the litters in groups A, B, C, and D, respectively (Table 2). Homologous protection against reproductive failure was demonstrated by a significant difference between groups A and B ($p = 0.0001$) and heterologous cross-protection was demonstrated by a significant difference between groups C and D ($p = 0.01$). No PRRSV was isolated from any gilts or pigs in Group E.

Serology --All gilts in group A and C developed PRRSV-specific antibody within 14 days of their challenge with either homologous or heterologous virus, respectively. No consistent evidence of an anamnestic humoral immune response against NADC-8 virus antigen was detected in group B following homologous challenge. All gilts in group D developed specific antibody against Lelystad virus antigen following heterologous virus challenge. Cross-reactivity against Lelystad virus antigen

was detected in one gilt, B-5. Cross-reactivity against NADC-8 virus antigen was detected in 4 gilts, C-3, 4, 5, and 8 (Table 2).

Discussion

In experiment 1 the conception rates were poor following insemination with semen containing either PRRSV or sham inoculum. There was no significant difference between the groups even though one group of gilts became infected with PRRSV around the time of conception. This result is similar to previous studies in which intrauterine inoculation of PRRSV at or near the time of conception did not significantly reduce conception rates (90, 145). The objective of experiment 1 was to immunize the gilts with field-type PRRSV approximately 90 days before challenge, an interval of time which is adequate for the development of homologous protective immunity (92). However, due to complications, some of the gilts were not challenged within the 90 day window resulting in a broad interval of time between immunization and challenge.

In experiment 2 homologous protective immunity, i.e., lack of detectable virus replication in the dam and lack of transplacental infection, was demonstrated in all gilts of group B. In contrast, no protective immunity was found in group A, i.e., evidence for virus replication with concurrent transplacental infection was found in each gilt. The interval of time between the immunizing dose of virus and challenge virus for group B ranged from 90 to 205 days. These results are consistent with 2 previous studies in which homologous challenge (NADC-8 virus) occurred 90 days (92) and 233 to 604 days (91) after the immunizing dose of virus and no evidence of virus replication in the dam or transplacental infection was found. Collectively, these homologous virus challenge studies are in agreement with vaccine studies in which the challenge virus was the parent strain of the vaccine (54, 66).

In litter A-8, the litter that aborted 20 days post challenge with NADC-8 virus, gross and microscopic fetal lesions were observed that are compatible with lesions observed in PRRSV-infected fetuses from a previous study (86). The presence of virus replication in gilt A-8, plus gross and microscopic fetal lesions, and the isolation of PRRSV from 4 of the 8 live-born fetuses suggest this litter was transplacentally infected and affected by the NADC-8 virus. In litter B-6, the litter that aborted 18 days-post-challenge with NADC-8 virus, no gross fetal lesions were observed and virus replication was not detected in the dam nor was transplacental infection detected in the litter. The cause of this abortion is unknown; although PRRSV as a cause cannot be ruled out, it seemed

unlikely based on previous experiences, absence of detectable virus in the dam or fetuses, and absence of fetal lesions.

In group C, the heterologous virus-exposed controls, 8 gilts had detectable virus replication and 7 of 8 had transplacental infection. One litter (C-3) did not have a detectable transplacental infection; this may be due to a failure to detect virus or the fetuses may not have been infected with Lelystad virus at the time of the gilt's necropsy. In our experience, this apparent lack of transplacental infection following field virus challenge at about GD 90 has occurred before, although it is at a very low incidence, i.e., 6 out of 126 naive, pregnant swine (5%) did not have a detectable transplacental infection following challenge at or about GD 90 with one or more PRRSV isolates (89, 91, 92, 107, 112).⁴ We assume the apparent lack of transplacental infection is due to chance, however, the Lelystad virus had 9 in vitro passages prior to its use for this study which may have resulted in some attenuation of the virus.

In group D, the heterologous protective immunity challenge group, 3 of the 8 gilts did not have detectable virus replication, i.e., 38% had protective immunity following challenge and 6 of the 7 pregnant gilts did not have transplacental infection, i.e., 86 % had cross-protection against transplacental infection. The results suggest heterologous protection from reproductive losses was induced by the immunizing dose of NADC-8 virus. Moreover, there may be a temporal effect because gilts challenged around 90 days after immunization (gilts D-1, 2, and 3) appeared protected, i.e., no detectable virus replication in the dam and no transplacental infection. However, the remaining gilts challenged 134 to 170 days after immunization appeared to have declining heterologous protective immunity based on the detection of replicating virus in gilts D-4, 5, 6, 7, and 8; and the detection of transplacental infection in gilt D-8.

Antigenic differences between North American and European PRRSV isolates have been demonstrated by IFA (6) immunoperoxidase monolayer assay (185), and monoclonal antibodies (126). The limited serologic cross-reactivity found in groups B (1 of 8 gilts) and C (4 of 8 gilts) was similar to previous reports in which the Lelystad virus induced some heterologous antibody while North American isolates produced little if any (186). Group D was exposed to two antigenically distinct viruses resulting in what we believe were two primary immune responses for each gilt. In addition, 4 of the 8 gilts developed a 4-fold rise in NADC-8 virus-specific antibody titer following the Lelystad virus challenge which suggested heterologous antibody may have been induced as seen in group C. However, if one considered Lelystad virus and NADC-8 virus to be antigenically similar, then the rise in NADC-8 titer may have represented a weak anamnestic immune response

since 3 of the 8 gilts in group B also developed a 4-fold rise in IFA titer. This possible weak anamnestic immune response is compatible with our previous observations in which few if any of the animals developed a detectable anamnestic humoral immune response following homologous challenge (91, 92).

In group D, it appears that the primary NADC-8 immune response may have attenuated the primary Lelystad virus immune response when virus-specific antibody titers are compared (group C vs. group D). This apparent suppression in Lelystad virus-specific antibody titer may reflect a suppression of Lelystad virus replication in the host previously immunized with NADC-8 virus. However, the number of animals in each group suggest additional studies should be completed to confirm these observations.

On the basis of this and previous studies (54, 68) it appears that exposure to one strain of PRRSV, regardless of its level of virulence, does not ensure complete protection against subsequent exposure to a heterologous strain. This lack of complete protection, and the related possibility for transplacental infection, suggests that vertical transmission of PRRSV may be an important, perhaps primary, means by which virulent PRRSV can be maintained even in a vaccinated herd. Therefore it seems likely that the success of any program designed for the control and eventual eradication of PRRS will depend on a combination of management practices, of which vaccination may be a single component.

It is possible that the partial failure of heterologous protective immunity described in this study, i.e., lack of heterologous protective immunity in the dam, is overstated because the NADC-8 virus and the Lelystad virus represent two different genotypes (103, 125), which are antigenically distinct (6, 126, 188). This difference should produce a rigorous test of heterologous immunity; which in this study was mostly a success, i.e., one isolate of PRRSV apparently induced a heterologous protection from reproductive loss against an antigenically distinct PRRSV. This success implies that vaccines derived from North American PRRSV isolates should be efficacious against antigenically similar PRRSV isolates; a supposition that is supported by one vaccine study (66).

In conclusion, we believe a homologous complete protective immunity was demonstrated in this study and a heterologous limited protective immunity was produced between two antigenically distinct viruses, the NADC-8 and Lelystad PRRSV isolates. The cross-protection may have a temporal effect in which the gilt is able to develop a protective immune response of limited duration followed by a decay in protection first recognized by detectable virus replication in the dam and then, by transplacental infection. We propose that the apparent limited protective immunity may be

related to the significant antigenic and genetic differences between the North American and European strains of PRRSV (103, 125), and to the observed phenomenon that PRRSV-specific antibody titer apparently decays over time following natural (2, 163) and vaccine^a induced immune responses. However, additional studies are necessary to confirm this putative temporal effect. Further studies are also necessary to investigate the possible additive effects of repeated vaccinations on the duration of heterologous protection.

^aKelly M. Lager, personal observations.

^bParvo Shield L5E, Grand Laboratories Inc., Larchwood, Iowa.

^cRegu-Mate, Hoechst-Roussel, Somerville, New Jersey.

^dLutalyse, Upjohn Company, Kalamazoo, Michigan.

^ePG 600, Intervet Inc., Millsboro, Delaware.

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CHAPTER 10. GENERAL CONCLUSIONS

PRRS was first recognized in 1987 as a few epizootics of reproductive failure and respiratory disease and within a few years it had spread into a pandemic. The rapid dissemination of this disease can be attributed to the dramatic changes that have occurred in the swine industry during the last 10 to 20 years, i.e., the concentration of animals at one site and the drive for genetic improvement. In the United States, for example, the construction of a barn that houses thousands of pigs or sows has become common. Moreover, the barns may be filled with animals from multiple sources or they may be continuously filled resulting in an almost perfect environment for promoting the evolution of pathogens. Most breeding herds have a high “turnover” rate (replacement of sows due to culling or improvement of genetics) resulting in 25 to 50 percent of the sow herd being replaced every year. This high turnover rate not only provides a continual source of animals naïve to endemic disease on the farm, but it also makes these herds vulnerable to the dissemination of disease by way of infected replacement gilts. In addition to these risks, there has been the almost exponential use of boar studs to supply semen to multiple farms; again, these husbandry changes can promote the transmission of disease, sometimes across great distances. The lack of knowledge about the pathogenesis and epizootiology of an infectious agent compounds these changes in swine husbandry. This has been clearly demonstrated in the case of PRRS since the etiologic agent was not discovered until 1991, about 4 years after the disease was first recognized.

The collection of work recorded in this dissertation represents studies beginning in 1992 soon after the discovery of PRRSV and culminating in 1997, a span of 5 years in which all major swine research laboratories in the world have investigated many aspects of PRRS. The focus of this dissertation, the pathogenesis of PRRSV-induced reproductive failure, mirrors the focus of the laboratory where the studies were completed, the Virology Swine Research Unit, National Animal Disease Center, USDA-ARS. The goal of this dissertation is to expand the knowledge base of PRRS so veterinarians may be able to apply this information to the control and prevention of this disease.

The major clinical signs associated with a PRRS epizootic were and still are reproductive failure and respiratory disease. The reproductive failure was believed to be a consequence of PRRSV infection of pregnant animals resulting in 1) abortions that were reported during all stages of gestation, however, the vast majority occurred during the last third of gestation; 2) early farrowings (delivery of weak and stillborn pigs from GD 108 to 112) and a significant increase in the incidence

of weak and stillborn pigs at term; 3) an increased incidence of dead and mummifying fetuses in subsequent litters; and 4) an increased incidence in returns to estrus and delayed returns to estrus. In general, the respiratory disease was most pronounced in the farrowing house and nursery resulting in high mortality rates that persisted through the grower and finisher phases of production. All of the clinical signs of reproductive failure and most of the respiratory disease were attributed to the direct effect of PRRSV on the host, however, some of the morbidity and mortality associated with PRRSV-induced respiratory disease were believed due to secondary bacterial infections that developed because of the putative PRRSV-induced immunosuppression.

The ability to culture PRRSV enabled investigators to initiate classic pathogenesis studies, i.e., administer virus to susceptible animals and observe them for virus-induced effects. Since late-gestation reproductive failure was the predominant clinical sign reported during PRRS epizootics the early pathogenesis studies quite naturally involved challenge of gilts or sows late in gestation (about GD 90).

The intra- and oronasal routes of infection were chosen because it was assumed naturally infected swine were susceptible to infection via a virus/host cell interaction on a mucosal surface of the respiratory tract and/or the oropharynx. The clinical signs of PRRS late-gestation reproductive failure were reproduced with some of the pregnant animals farrowing before predicted delivery times while others farrowed on schedule or were late (13, 15, 25, 35, 107, 112, 135, 175, 197). Essentially all litters from experimentally infected animals were affected in some way with either weak pigs, stillborn pigs, or dead fetuses at the time of farrowing and virus was detected in tissues or sera of some liveborn, stillborn, and dead fetuses indicating transplacental transfer of virus had occurred. Some of the pigs and dead fetuses also contained PRRSV-specific antibody at birth indicating some level of fetal immunocompetence against PRRSV (15, 107, 112). In addition to reproductive failure some of the gilts and sows developed moderate pyrexia, anorexia, and depression within several days of exposure that had a variable duration. These early investigations have been followed with several studies into the pathogenesis of early- and mid-gestation PRRSV-induced reproductive failure.

Experimental PRRSV infection at the time of breeding did not have any significant effect on conception (144, 145). However, PRRSV infection at the time of breeding or at GD 14 did result in some embryonic infection and death (140, 144, 145). In 2 studies investigating mid-gestation PRRSV infection (GD 45 to 50) transplacental infection was not immediately identified in fetuses examined 1 to 6 weeks PE of the dam although it did occur in two litters that went to term (22) and in one litter examined 9 weeks PE of the dam (107).

In these early studies no micro- or macroscopic lesions were noted in dead fetuses, stillborn pigs, or weakborn pigs at birth although a mild endometritis was reported in one study involving late-gestation experimental infection (22). Subsequent studies have found myometritis, endometritis, and maternal placentitis in a field and experimental case (164) and a case report described vasculitis involving several major organs in fetuses aborted late in gestation (152). All of these lesions were attributed to a PRRSV infection.

Collectively, the observations from these early studies can be used to deduce certain aspects of the pathogenesis of PRRSV infection in pregnant swine and they will be discussed in conjunction with the work reported in this dissertation in an outline format describing clinical effects on the dam, abortion mechanisms, consequences of maternal infection during late-, mid-, and early-gestation, development of protective immunity in the sow, and transfer of passively acquired immunity in pigs.

Clinical signs of the dam -- The incubation period from experimental infection to clinical signs in the dam has been about 48 to 72 hours at which time some gilts and sows developed anorexia, pyrexia, and listlessness of variable magnitude and duration. This is important from a diagnostic standpoint since clinically affected gilts and sows are presumed to have been infected for at least several days and with the duration of viremia in adult swine being relatively short, the observation of clinical signs could almost correspond with the loss of detectable viremia thus making the timing of sample collection critical for successful virus isolation. In general, the maternal clinical signs reported in this dissertation have been compatible with previous observations in that they have also been mild. For example, on occasion experimentally infected gilts were anorexic, listless, pyrexia, and rarely, they developed cutaneous lesions compatible with "Blue Ear". The experimental infections appeared to mimic anecdotal field reports of high health-status sows infected with PRRSV in which few maternal signs of infection were noted except for the reproductive failure during late-gestation. Based on field reports it appears maternal clinical signs associated with PRRSV infection become more intense as pregnancy advances. Whether this is a true phenomenon or not is not known.

Abortion mechanisms -- Abortions were commonly reported during PRRS epizootics and when estimated the incidence has involved up to 4% of the sow herd. According to field observations, the vast majority have occurred during late gestation although they have been described during all stages of gestation (73). The emphasis on late-gestation abortion probably reflects both a true higher incidence of late-gestation versus early-gestation abortion and an underreporting of early-gestation

abortions due to several possibilities, e.g., small aborted fetuses/embryos may not be observed as readily or the dramatic effect of finding a pile of large fetuses on the floor may be more memorable and thus reported more frequently.

Aborted litters under field or experimental conditions have generally contained fetuses that all appeared the same, i.e., each one had minimal or no detectable autolysis indicating they had been alive at the time of abortion or they had all died about the same time *in utero*, usually just prior to expulsion. Occasionally some aborted litters had a few fetuses that appeared to have been dead longer than others based on gross appearances suggesting they had died at different times *in utero*, these instances have involved late-gestation litters. Based on discussions with diagnostic laboratories about PRRS abortion cases, isolation of PRRSV from fetuses aborted prior to GD 90 is a very rare event and virus is seldom isolated from fetuses aborted after GD 90. Our field experience at NADC involving virus isolation from fetuses aborted after GD 90 has been much more promising; however, it is still a very rare event to isolate PRRSV from fetuses aborted prior to GD 90.

Analysis of these observations and data supports several assumptions: 1) if PRRSV was the causal agent for these abortions, then fetal infection does not appear to be a prerequisite for abortion; 2) lack of transplacental infection in aborted fetuses could be due to abortions occurring prior to transplacental infection; and 3) the apparent higher frequency of virus isolation from fetuses aborted during late-gestation compared to early- or mid-gestation may be due to a selective permeability of the placenta for PRRSV as hypothesized by Christianson et al. (22).

Evidence for a maternal factor responsible for abortion is supported by unpublished observations, i.e., the description of multifocal areas of necrosis associated with inflammation in the corpora lutea (CL) of PRRSV-infected gilts and sows that is not observed in non-PRRSV infected controls (Personal communication, Dr. Pat Halbur, ISU-VDL). Assuming these lesions affect the progesterone secreting properties of the CL, then a loss of progesterone could lead to abortion resulting in aborted non-autolytic fetuses that are either alive or had died just prior to expulsion. This hypothesis would account for the abortions that have been reported in which normal appearing fetuses free of autolysis and PRRSV infection were found. The lack of transplacental infection could be explained by abortions occurring prior to transplacental infection, an event that can be estimated based on several studies.

As discussed previously in chapter 2, the time interval from exposure of the dam to transplacental infection during late-gestation has been deduced based on the fact that late-gestation fetuses are immunocompetent to PRRSV and it is assumed that fetuses can produce antibody within 14 days of

infection. Therefore, in the case of gilts experimentally infected at GD 90, seropositive pigs at birth (114 days of gestation) would have been infected with PRRSV by at least 100 days of gestation, or 10 days PE. Based on this assumption, if abortions occurred within the first 10 days after infection of the dam, then one would expect a low rate of fetal infection because the fetuses have yet to be infected and the cause of abortion is failure of maternal maintenance of pregnancy rather than infected fetuses that die and then initiate the abortion process. In the studies reported here the earliest transplacental infection following late-gestation challenge of the dam was documented by isolation of virus from fetuses aborted 12 days PE of the dam in chapter 8 when 1 gilt aborted following PRRSV challenge and some of the litter was transplacentally infected. In appendix B, no transplacental infection was detected in 3 litters examined 7 days PE of the dam at GD 90, although all 3 litters at 14 and 21 days PE of the dam had transplacental infection. These studies support the assumption that transplacental infection could occur around 10 days PE of the dam during late-gestation. Abortions were noted in chapter 7 where 2 of 12 gilts were known to have aborted about 7 to 10 days after PRRSV challenge at GD 30, no virus was detected in these early/mid-gestation aborted fetuses. Two additional gilts from that group may have aborted following challenge although no physical evidence was found. It is assumed these abortions were due to the maternal PRRSV infection.

The earliest indication of transplacental infection following infection of the dam reported in this dissertation was in 2 gilts exposed to PRRSV at GD 30 as described in appendix B. PRRSV was isolated from the amniotic fluid of some of the fetuses examined 7 and 8 days PE of the dam. Some gilts and sows experimentally infected at or about GD 90 aborted transplacentally infected fetuses (chapters 7 to 9) indicating the dams had been infected with virus for at least several days prior to abortion to account for the infected fetuses. Whether these “delayed” abortions would be attributed to the putative maternal dependent abortion mechanism (ovarian dysfunction) that is hypothesized for the abortion of non-transplacentally infected fetuses or to some fetal initiated cause is unknown.

In contrast to the abortions described in experimental studies there have been examples of prolonged gestation ending with the delivery of a litter consisting of dead fetuses, most of which were undergoing a mummification process (112). The lack of abortion despite death of the entire litter suggests that fetal death by itself does not initiate expulsion and the prolonged gestation implies that there may even be an absence of fetal signals for initiation of parturition.

It is possible the etiology of the abortions is not dependent on ovarian dysfunction, but rather it is due to uterine inflammation that has been described infrequently for experimental and field cases (22, 164). This uterine inflammation could cause a loss of placental integrity and function that would be compatible with what was observed in the studies reported here, i.e., the appearance of normal PRRSV-infected fetuses that had obvious macroscopic lesions involving the placenta (chapter 6).

The proposed abortion mechanism(s), i.e., virus-induced necrosis of the CL and uterine inflammation, could explain why abortions occur, but not necessarily the incidence rate since only a small percentage of infected animals do abort in the field (or in experiments) while most if not all of the herd was presumed infected. One could assume the animals that did not abort did not have significant lesions involving the CL or uterus, significant at least from the standpoint of causing an abortifacient decrease in progesterone or uterine function. Moreover, it is possible that these two proposed mechanisms could have synergistic interactions resulting in abortions.

Late-gestation reproductive failure (GD 76-114) -- Transplacental infection indicates PRRSV has replicated in the dam and crossed the placental barrier; this has occurred almost 100% of the time following exposure to a PRRSV field isolate in the GD 90 experimental challenge model. If the infected dam farrows the litter will usually be a mixture of live pigs, stillborn pigs, and dead fetuses. Some of the live and stillborn pigs and even some of the dead fetuses will contain PRRSV-specific antibody while others are infected and have not yet seroconverted. These litters have provided some details about the temporal aspects of transplacental infection since some fetuses became infected, seroconverted, and died prior to farrowing, about 24 days PE of the dam. Most of the fetal deaths appeared to have occurred within a few days of parturition (about 3 weeks PE) providing the basis for the timing of necropsies in the studies reported in this dissertation involving GD 90 challenges followed by necropsy of the dam.

The clinical effects of PRRSV infection on naive pregnant gilts and sows described in chapters 6 to 9 and appendix B and C are in agreement with previous studies. First, as would be expected in a biological system, a broad spectrum of clinical signs was observed in the litters of these experimentally infected dams. Usually in these litters about 3/4 of the fetuses were alive at the time of necropsy with about half of them infected with PRRSV. Most of the dead fetuses were estimated to have died within 2 days of necropsy based on the minimal amount of autolysis observed, although some had been dead for a longer period of time based on extensive autolysis and the beginning of the

mummification process. The estimated time of fetal death was made with some confidence based on an earlier study that dealt with fetal postmortem changes *in utero* (87). The observation of dead fetuses 21 days PE that had been dead for several days *in utero* suggests the fetuses may have died 17 to 18 days after the dam's exposure to virus. Some of the live and dead fetuses tested 3-weeks-PE of the dam had developed PRRSV-specific antibody indicating they had been infected and developed a detectable immune response that in the case of the dead fetuses, was certainly not protective. Whether the presence of antibody in live fetuses indicates some protection or the live seropositive fetuses were just sampled prior to death is unknown. Antibody in some but not all of the dead fetuses suggests that some immune-mediated event does not play a pivotal role in fetal death, e.g., ADE or some other immunopathologic mechanism. Probably, the presence of antibody in some dead fetuses indicates the time course from fetal infection to death could be variable, and in some fetuses death may take 10 to 14 days or longer allowing for the development of an immune response prior to death. Why some fetuses become infected, seroconvert and die while others become infected, seroconvert and survive to parturition is unknown.

As reported in chapter 6, the experimental model for late-gestation reproductive failure consistently produced litters containing live and dead fetuses that represented a spectrum of PRRSV-induced fetal affects summarized here as live normal fetuses that may or may not have been transplacentally infected, live fetuses abnormal in appearance that were infected with virus, fetuses that had recently died from which virus could be isolated and fetuses that were obviously undergoing postmortem autolysis from which virus could not be isolated. This spectrum of fetal changes suggests a series of events follow fetal infection during late-gestation. These fetal changes can occur in a relatively short period of time, i.e., a few days to a couple of weeks following infection.

A set of gross lesions was commonly observed in PRRSV-infected fetuses examined 21 days PE of the dam that probably reflect both direct and indirect effects of the virus. Direct effects would be attributed to cytolytic properties of the virus, e.g., the macroscopic damage to the umbilical cord is probably due to a loss of vascular integrity because PRRSV affected the umbilical arteries. Indirect effects could be the edema observed within the abdominal cavity that is probably secondary to vascular damage or cardiac insufficiency resulting in the development of edema around the kidney, pancreas, and in the mesentery of the spiral colon and the accumulation of an effusion in the thoracic and abdominal cavities.

Microscopic examination of fetal tissues supported the gross observations of PRRSV-induced vascular damage because inflammatory lesions had a lymphoplasmacytic character compatible with

a virus infection and were associated with the vasculature (chapter 6). In addition to the umbilical cord lesions reported in chapter 6 a vasculitis was observed in other fetal tissues that is similar to vascular lesions described in a case report on aborted fetuses collected from field cases diagnosed as PRRS reproductive failure (152). The experimentally-induced lesions appeared to be associated with arterial blood vessels and were analogous to lesions described in mares and their fetuses that were attributed to EAV infection (75).

There is limited knowledge about any microscopic placental lesions that may offer insight into the mechanisms for the loss of placental integrity. In the studies reported in chapters 3, 4, and 6 to 9 and in appendix B and C, gross lesions were observed in many dead fetuses experimentally infected with PRRSV, however, not all dead fetuses infected with PRRSV had lesions. Occasionally some normal appearing live and dead fetuses infected with PRRSV had gross lesions associated with their placenta. Three conditions have been observed infrequently: 1) multifocal diffuse hemorrhages in the fetal placenta; 2) the placenta and uterus were edematous; and 3) portions of the fetal placenta were a tannish-white color and have detached from the uterus. In all 3 conditions, the fetuses were infected with PRRSV, normal in appearance, and most were alive or estimated to have died very recently. These observations suggest the placenta was affected by PRRSV leading to a loss of function resulting in the death of a normal appearing fetus. It is not known if these descriptions of placental lesions reflect 3 separate outcomes of PRRSV infection or if they are just “snapshots” of progressive lesions. Microscopic examination of the uterus and placenta of gilts exposed to PRRSV 21 days previously revealed a mild endometritis, myometritis, and occasional placentitis, lesions that were compatible with previous observations by others (22, 164).

Based on the studies reported here and the work of others, transplacental infection does occur and some of the infected fetuses do die within 2 to 3 weeks of exposure of the dam with PRRSV during late-gestation. What is less clear though, is the cause of fetal death. At a macroscopic level it appears to be related to a loss of vascular integrity or placental function, both could be diffuse events that may not have any focal lesion except for segmental circumferential necrosis in umbilical cords. At a microscopic level there may be a singular underlying cause to the previously described lesions that would involve the cytolytic affects of PRRSV on smooth muscled arteries similar to what has been described for EAV (75). This arteritis would certainly explain the affected umbilical cords, fetal lesions, and could account for placental dysfunction.

Mid-gestation reproductive failure (GD 39-75) -- As stated previously, in the field reproductive failure during mid-gestation is described less frequently than during late-gestation. Based on experimental studies, mid-gestation reproductive failure does occur at a lower incidence when compared to late-gestation. The reason for any difference is unknown, however, a hypothesis can be deduced based on data from this dissertation and the works of others.

Swine develop a viremia soon after infection with PRRSV that, depending on the age of the animal, may persist for at least several weeks. In addition to the apparent free virus circulating in the blood is the presence of cell-associated PRRSV that is believed to be either attached to or residing in circulating cells of the phagocytic lineage. The exact *in vivo* concentration of these putative circulating PRRSV-infected cells and how long this population may exist, especially in a pregnant sow, are unknown; however, several assumptions can be made based on observations.

Although the duration of this putative population of infected cells is unknown it may be estimated to last at least several weeks or longer based on the observation that PRRSV-specific nucleic acid can be detected in the cellular fraction of semen for up to 92 days PE of boars (27) and in the buffy coat of congenitally infected pigs for up to 210 days post-partum (10). In both instances viral nucleic acid is considered presumptive evidence for the presence of virus implying cell associated PRRSV can persist for many weeks or months after the cessation of viremia.

A rough estimate of the incidence of transplacentally infected fetuses can be made based on the assumption PRRSV crosses the placental barrier associated with a maternal cell and at least one infectious virus particle is needed for infecting a fetus and at least one maternal cell is necessary to transfer one virus particle.

In chapters 3, 7, 8, and 9 and appendix B gilts and sows were necropsied at selected times after experimental infection during late-gestation. Although the incidence of infected fetuses per litter was variable (ranging from 1 to all fetuses in a litter) most litters had about 1/2 of the live fetuses infected. In litters that had fewer infected fetuses it was common to see a single infected fetus bordered by non-infected fetuses or just a few infected fetuses in only one horn of the uterus. These observations implied that only a few of the fetuses were initially infected in the first waves of maternal virus replication and the rest of the fetuses became infected by intrauterine spread of virus or by subsequent transuterine migration of virus-infected maternal cells. Assuming the susceptibility of fetuses and pigs to PRRSV infection is similar, then a plausible explanation for the low incidence of transplacental infection during the acute stage of maternal infection in late-gestation would be that only a few virus-laden maternal cells made the transuterine migration and established fetal infection.

Experimentally infected litters at 3-weeks-PE and at the time of farrowing contain a spectrum of normal and abnormal infected fetuses that may be attributed to several causes: 1) all of the infected fetuses were transplacentally infected at the same time and they succumbed to infection at different rates; 2) infected fetuses succumb to infection at the same rate, but they became transplacentally infected at different times; and 3) infected fetuses succumb to infection at the same rate, but some of the fetuses were transplacentally infected and then spread virus to littermates by intrauterine transmission resulting in fetuses infected at different times. With all this in mind the chance of an individual fetus becoming transplacentally infected late in gestation is apparently low; however, the chances are additive and the probability of at least one fetus in a litter becoming transplacentally infected is high, especially for large litters.

The explanation for only a few fetuses infected during the acute stage of maternal infection could be the hypothetical maternal cells responsible for carrying virus across the placental barrier exist in a limited population. On the other hand, the maternal cell population could be large and it is a very rare event for infected cells to exit the maternal circulation and migrate into fetal circulation. Both scenarios attempt to explain the presence of a few fetuses infected during the acute phase of infection. Of course there is biologic variation resulting in the occasional litter where all the fetuses in a litter were infected about the same time and maybe all of them even died *in utero* about the same time due to either direct virus effects on the fetus or indirect effects like a generalized loss of placental integrity.

Why is there an apparent difference in the incidence of reproductive failure following PRRSV infection during mid- or late-gestation? One hypothesis suggested that the placenta was less permeable to virus during mid-gestation when compared to late-gestation (22). This permeability was based on a decrease in the anatomic distance between maternal and fetal circulation that occurs as gestation progresses due to the capillary beds in the fetal placenta expanding into the trophoblast. Another possibility is that the anatomic barrier has little to do with the chances for transplacental infection; rather, as previously discussed the putative PRRSV-infected maternal cell emigrating from maternal circulation in the uterus is a random event resulting in a low probability of infecting a single fetus. The reason for a higher incidence of transplacental infection during late-gestation compared to mid-gestation may simply be dependent on the dramatic increase in uterine blood flow as gestation progresses. The increased blood volume may increase the chance that a rare event could occur, i.e., emigration of a PRRSV-infected cell from a uterine capillary bed into maternal tissues and through the placenta into fetal tissues resulting in fetal infection. Assuming large litters would

require more uterine blood flow than small litters, then this hypothesis is supported by 2 litters in chapter 8 that did not have detectable transplacental infection following challenge of the dam at GD 90, one contained 2 fetuses and the other 5 fetuses. Of course a combination of these two factors, i.e., the thinning of the anatomic barrier in conjunction with the increased blood flow could increase the chances of maternal cells crossing the placental barrier resulting in a higher incidence of transplacental infection during late-gestation.

A third factor that may also play a role in the incidence rates of clinical reproductive failure is the apparent age-based susceptibility of fetuses to PRRSV. Chapter 3 describes a study where fetuses directly infected at GD 85 responded as would be anticipated, i.e., they replicated virus and began to die within 2 to 3 weeks of inoculation. In contrast, fetuses infected similarly at GD 34 replicated virus and were alive and normal in appearance 21 and 31 days post inoculation. Moreover, fetuses inoculated between GD 45 to 49 appeared to replicate virus and survive until about GD 65 to 70 at which time some of the fetuses were beginning to succumb to PRRSV infection. This study demonstrated fetuses were capable of virus replication from GD 34 and early- and mid-gestation fetuses (GD 34 to 49) appeared less susceptible to the immediate lethal consequences of PRRSV when compared to late-gestation fetuses. Why the fetuses begin to die is unknown. It appears they support virus replication for extended periods of time in some cell population(s) without producing any gross lesions. However, once the infected fetuses approach 65 to 70 days of gestation, they develop gross lesions and death appears imminent. The lungs appeared the most affected and microscopic study revealed necrosis of the bronchial buds and subjacent smooth muscle. In addition, there was significant hemorrhage in the interstium of the lung. It is unknown exactly which cell type(s) were involved and it is assumed the lesions reflected direct viral damage as opposed to some indirect affect.

In chapter 7 gilts exposed to PRRSV at GD 30 had a high incidence of fetal death around GD 70 that was attributed to PRRSV since 2 of the litters had a known transplacental PRRSV infection. In Appendix B transplacental infection was detected in 6 of the 23 gilts exposed to PRRSV at GD 30; in the litters examined at less than GD 70 all of the infected fetuses appeared normal and after GD 70 some of the infected fetuses were affected and had either died or death appeared imminent. Collectively, these mid-gestation studies indicated that fetal death was less common when compared to late-gestation. This difference may delay the development of maternal clinical signs recognized as PRRS.

There are few reports of reproductive failure occurring during mid-gestation in the field despite the high probability of sows becoming infected at this stage of gestation and the apparent susceptibility of mid-gestation fetuses. However, in retrospective analysis some of the veterinarians and producers do recall pregnant animals potentially exposed to field virus during early- and mid-gestation that had reproductive losses at or near term that were attributed to PRRSV. These losses were usually expressed as decreases in the average number of live-born pigs per litter attributed to an increase in mummies and late-gestation fetal death. In general, most of the live born pigs were normal suggesting they may not have been infected with PRRSV. The clinical scenario just described can be explained based on the findings in chapter 7 where a group of gilts were oronasally exposed to PRRSV at or about GD 30. The gilts were examined 81 days PE and a high incidence of large mummies was found suggesting this fetal death could be attributed to PRRSV. However, no virus was isolated from the mummies or the live-normal littermates. Two litters of this group described in chapter 7 did have transplacental infection. Each litter contained mummies and one viremic, seropositive fetus. The position and size of the mummies in each uterus suggested each fetus started to die at different times, probably sometime after GD 65 based on crown-rump length of the mummified fetus.

Early-gestation reproductive failure (GD 1-38) -- Chapters 5 and 9, studies involving intrauterine administration of PRRSV, support the work of others indicating the sow could be susceptible to venereal transmission of PRRSV and the intrauterine administration of virus had no significant effect on conception. Moreover, the litters from gilts in these studies were examined at 111 days of gestation and no difference in litter size was found suggesting the intrauterine exposure to PRRSV near the time of conception did not significantly affect conception or the development of the fetuses. These observations are in slight disagreement from the work of Prieto, et al. that involved infecting the dam near the time of conception or at GD 7, 14, and 21 (140, 144, 145). Results of these studies indicated PRRSV infection did not affect conception, however, it did cause embryonic infection between GD 10 and GD 20 that presumably would cause reproductive failure. The observations of infected and affected embryos could explain the field reports of irregular late returns to estrus that have been attributed to PRRS; however, the failure to experimentally reproduce conception failure does not support the role of PRRSV as a cause of increased regular returns to estrus. The findings of no affect on conception rate in chapters 5 and 9 are in agreement with the conclusions of Prieto, et al. If increased regular returns to estrus are related to PRRS, then the cause may be paternal, i.e., poor

semen quality. However, if PRRSV does affect ovarian function, then increased regular returns to estrus may be related to when the dam is infected with virus, e.g., prior to breeding may disrupt fertility of the dam. It is necessary to remember that the number of animals in most PRRSV studies is small and the significance of any observations should not be overstated, especially in light of the fact that there may be dramatic differences between PRRSV strains.

Based on experimental studies reported in chapter 7, appendix B, and by Prieto, et al.; infection of the dam around the time of the development of the placenta or soon thereafter (GD 15-30) could result in fetal infection which supports the role of infected maternal cells migrating across the placenta and establishing fetal infection as opposed to PRRSV just growing through the uterine epithelium and flooding the uterus with virus.

Development of protective immunity in the sow -- Based on field observations swine are believed to develop protective immunity following natural infection that lasts for an undetermined amount of time. Homologous and heterologous immunity have been reported following vaccination of gilts with MLV prior to breeding (54, 66, 68). The immunized gilts were challenged during late-gestation with either the virulent parent strain of virus (homologous virus) resulting in complete protection of fetuses or with a heterologous strain that caused some transplacental infection although the magnitude of reproductive failure was significantly reduced when compared to non-vaccinated challenge control gilts.

Chapters 7 to 9 and appendix C discuss the development of protective immunity in sows following immunization with field virus or MLV. These studies essentially support field observations of protective immunity and confirm the MLV reports from biologics companies. In addition, they suggest that homologous protection following experimental immunization could develop within at least 90 days of exposure (chapter 7) and it could last for at least 600 days (chapter 8). However, this may not be the case for heterologous protective immunity since some immunized animals are not fully cross-protected for reasons that are unknown (chapter 9 and appendix C).

The character of the protective immune response is unknown. It is assumed cell mediated immunity plays a major role in the protective immune response and this is supported by limited studies that have demonstrated antigen specific T-cell responses (9). Humoral immunity does develop following infection although the significance of this immune response is in question since virus can circulate in the presence of antibodies for at least several weeks. Neutralizing antibodies can be detected within 2 to 4 weeks of infection. Usually, they coincide with the beginning of a

decrease in detectable viremia, however, the development of a measurable T-cell response (9) occurs at about this same time, thus confusing the role of antibody in the clearance of virus. Although PRRSV-specific antibodies probably have a role in protective immunity, the potential role of antibodies in ADE can not be ruled out.

Limited data is available about the immune response to a commercially prepared KV that has been reported to be efficacious against the reproductive form of PRRS based on the reduction of non-viable pigs at parturition following challenge of vaccinated sows (21). It is assumed the KV would stimulate a predominately humoral immune response with only limited involvement of cell mediated immunity; however, none of this has been evaluated.

Transfer of passively acquired immunity in pigs -- Preliminary studies investigating passively acquired immunity have reported no viremia and little or no clinical response following PRRSV challenge of pigs that have suckled seropositive dams (56, 124). The lack of viremia and clinical response was interpreted as protection from the deleterious effects of PRRSV due to passively acquired antibody. Similar observations were made in the studies discussed in appendix D in which pigs with passively acquired antibody were challenged with either a homologous or heterologous PRRSV strain (heterology defined as a different virus strain from what the sow had been exposed to before breeding).

Except for one pig in trial A of appendix D, no viremia or clinical response was identified in challenged pigs. The pigs were necropsied at 7 weeks PE and all appeared normal and free of virus. The one exception was a weak pig from the homologous challenge group that was euthanized at 1 week PE, it was not viremic, however, virus was detected in its lung lavage. This phenomenon of colostral antibody inhibiting the isolation of virus from serum also was reported in appendix C. Based on these findings it was assumed that all pigs in both challenge groups may have replicated virus to some degree but not to a level that stimulated any detectable humoral immune response.

In trial B, contact control pigs were placed into the pens with the objective of detecting virus shedding from the challenged pigs. At least one challenged pig in both challenge groups shed virus to contact pigs based on the contact pigs seroconverting and having virus-positive lung lavages. Interestingly the challenge pigs in both groups developed a humoral immune response when compared to non-challenge control pigs. Moreover, virus was detected in lung lavages collected from both groups of pigs at necropsy 6 weeks PE indicating these pigs may have been infected with

virus from the contact controls since no virus was detected in the lung lavages of challenge pigs in trial A.

In trial C contact control pigs were again used to detect virus shedding. In addition, the virus-challenged pigs were euthanized at weekly intervals following challenge with the objective of detecting the onset of virus replication. Three of the 4 challenged pigs were replicating virus in their lungs 1 week post challenge (appendix D, table 1) suggesting the challenge virus was replicating in them as opposed to the challenged pigs becoming reinfected by the contact controls as hypothesized in trial B. Although the number of animals is very small it appeared the last challenge pigs to be euthanized were developing a humoral immune response when compared to the non-challenged control pigs in trial C. Apparently the contact control pigs for the homologous virus challenged group did not become infected which is surprising compared to the results of trial B and the heterologous group contact controls in trial C. A possible explanation is that homologous antibody may inhibit shedding of virus to some degree resulting in a lack of transmission in the homologous challenge group in trial C.

Collectively the observations recorded in appendix D suggests passively acquired antibody had minimal protection against PRRSV infection; however, the pigs appeared normal following infection and it is possible that passively acquired antibody may have some protection against clinical disease. On the other hand healthy pigs usually appear normal following infection with most strains of PRRSV and limited clinical signs would have been anticipated. Further studies are certainly warranted to investigate the effects of passively acquired antibody.

In summary the information presented in this dissertation supports previous studies describing PRRSV as a primary swine pathogen. The pathogenesis studies give insight into the clinical presentation of field cases and provide data for selection of the best diagnostic samples. The protective immunity studies confirm studies completed by vaccine companies and indicate that some cross-protection is possible although MLV are not perfect, i.e., they do not completely prevent the onset of clinical disease in some vaccinated animals following challenge. The knowledge gained by these studies can contribute to the implementation of control programs.

APPENDIX A: EVALUATION OF PRRSV TRANSMISSION RISK FROM GILTS AND SOWS PREVIOUSLY INFECTED WITH PRRSV TO NAIVE ANIMALS IN DIRECT CONTACT

Five separate trials designed to evaluate the risk of PRRSV transmission from gilts and sows to age-matched contact control animals are recorded in this appendix and summarized in table 1. In trials A-D gilts and sows were experimentally infected with field virus, and in trial E gilts were vaccinated with a MLV.

Trial A -- At weekly intervals two gilts each received an intrauterine exposure to a PRRSV field isolate (n=16). Each pair of gilts were housed in isolation facilities for 3 weeks PE at which time they were then moved outside and housed as a group for the duration of this trial. One day after the last pair of gilts were moved to the outside pen (day 0 of the trial), a boar free of PRRSV-specific antibody was moved into an adjacent pen with fenceline contact. He was allowed into the gilt pen twice a day for estrus detection and breeding purposes. He remained in contact with the gilts for 57 days and was seronegative at the conclusion of this trial.

Trials B, C, and D -- Each of these trials had a similar design utilizing animals from 3 experiments investigating the pathogenesis of PRRSV-induced reproductive failure. Gilts were oronasally exposed to PRRSV at or about GD 90 of their first pregnancy in isolation facilities and allowed to farrow. Surviving pigs were weaned at 2 to 3 weeks of age and the now PRRSV-exposed sows were moved to outside facilities and commingled with the non-infected control sows from the respective study. All sows were housed together and were exposed to a PRRSV negative boar as previously described for breeding purposes. Each group of sows were housed together until approximately GD 90 at which time the transmission risk trial was concluded and the sows were utilized for an additional study. None of the contact sows or boars developed PRRSV-specific antibody in trials B, C, and D.

Trial E -- Gilts were housed outdoors in a pen and vaccinated on day 0 and 28 of this trial with a commercially available MLV. On day 57, 16 naive gilts and 2 boars were moved into adjacent pens with fenceline contact in the following order; vaccinated gilts-boar-boar-nonvaccinated gilts. The boars randomly bred gilts from both groups as they came into estrus. Although there was no direct

contact between groups of gilts in this trial, the boars had direct contact with both groups. None of the non-vaccinated gilts or boars developed PRRSV-specific antibody in this trial.

Table 1: Number of animals in transmission studies A-E.

Trial	# of PRRSV ^a exposed animals	# of contact sows	# of contact boars	Days between exposure and contact ^b	Duration of contact ^c
A	16	0	1	22 - 71	115
B	12	4	1	42 - 48	115
C	8	2	1	42 - 48	115
D	12	4	1	42 - 48	90
E	20	0	2	56	210

^aAnimals in trials A-D exposed to field PRRSV and in trial E exposed to MLV. ^bNumber of days between first exposure of animal to PRRSV and the beginning of contact trial. ^cApproximate number of days animals were in contact.

**APPENDIX B: GILTS ORONASALLY EXPOSED TO THE NADC-8 PRRSV FIELD
ISOLATE AT 30 OR 90 DAYS OF GESTATION AND NECROPSIED
AT WEEKLY INTERVALS THEREAFTER**

Thirty-three pregnant gilts free of PRRSV-specific antibody were used for this study. Gilts were exposed to the NADC-8 isolate of PRRSV at either GD 30 (n = 23) or at GD 90 (n = 9) and at weekly intervals thereafter 3 gilts per group were euthanized and selected tissues and fetal sera tested for PRRSV (table 1).

Transplacental infection was detected in 6 of 23 gilts and 6 of 9 litters following virus challenge of the dam at GD 30 and 90, respectively. Lung lavage was the most sensitive tissue to detect virus replication for up to 8 weeks PE of the dam. These results are compatible with earlier studies that demonstrated the incidence of transplacental infection is greatest during late gestation and the lung lavage is a superior sample for virus detection when compared to serum and tonsil (107, 112).

Table 1: Results of virus isolation performed on individual gilt tissues and the presence of transplacental infection for each gilt.

Sow #	Day of gestation		Virus Isolation ^a					
	Exposure ^b	Necropsy ^c	Lavage	Serum	Tonsil	Tonsil Scrape	Lymph Node	Transplacental Infection ^b
29	30	37	+ ^e	-	-	+	+	Yes
30	30	38	+	+	-	-	+	Yes
31	30	44	+	-	-	-	-	No
35	30	45	+	-	+	+	+	No
36	30	45	+	-	-	-	-	No
37	30	51	+	-	-	-	+	No
38	30	51	+	-	+	-	+	Yes
39	30	51	+	-	+	-	-	No
137	30	58	+	-	-	-	-	No
32	30	58	+	-	-	-	-	No
119	30	60	+	-	-	-	-	Yes
108	30	64	-	-	-	-	-	No
12	30	65	-	-	-	-	-	Yes
11	30	66	+	-	-	-	-	No
14	30	72	+	-	-	-	-	No
33	30	72	-	-	-	-	-	No
115	30	73	-	-	-	-	-	No
34	30	79	+	-	-	-	-	Yes
3	30	79	+	-	-	-	-	No
23	30	79	-	-	-	-	-	No
13	30	86	+	-	-	+	-	No
21	30	86	+	-	-	-	-	No
22	30	86	-	-	-	-	-	No
24	90	97	+	+	+	+	+	No
26	90	97	+	-	+	+	+	No
20	90	97	+	+	+	+	+	No
127	90	104	+	-	-	-	-	Yes
139	90	104	+	-	-	-	+	Yes
27	90	104	+	-	+	-	+	Yes
145	90	111	+	-	-	+	+	Yes
28	90	111	+	-	-	-	-	Yes
25	90	111	+	-	+	-	-	Yes

^aVirus isolation conducted on sow tissues collected at necropsy -- lung lavage, serum, tonsil tissue, scraping of tonsillar epithelium, uterine lymph node. ^bDay of gestation when gilt oronasally exposed to PRRSV; i.e., 30 = 30-31 days of gestation, 90 = 89-91 days of gestation. ^cDay of gestation when gilt was necropsied. ^dTransplacental infection defined as the detection of virus in serum of one or more fetuses in the litter; amniotic fluid was tested for litters 29 and 30. ^eVirus isolation results reported as positive (+) or negative (-).

APPENDIX C: EVALUATION OF CROSS-PROTECTION AMONG ANTIGENICALLY SIMILAR PRRSV ISOLATES

Twenty-five sows assembled from previous experiments were naturally bred. At or about GD 90 they were divided into one of two groups based on their previous history. Sows in group A (n = 6) were non-infected controls from previous experiments with no history of exposure to PRRSV. Sows in group B (n = 19) had been experimentally exposed once to one of several PRRSV isolates 28 to 42 days before breeding. On GD 90 each sow was moved into isolation facilities, blood was collected just prior to an oronasal exposure of challenge virus and again at 7 days-post-challenge and at farrowing. Pigs were bled prior to suckling and a sample of colostrum was collected from the sow prior to contact with her pigs. At 6 to 7 days post-partum all piglets and sows were euthanized and a blood sample and lung lavage were collected from each animal. Sera and lung lavages were tested for PRRSV and PRRSV-specific antibody as previously described.

A PRRSV protective immune response in the sow was defined as a lack of detectable PRRSV in the sow at the time of necropsy (approximately 30 days post-virus challenge) determined by testing the lung lavage for virus, the most sensitive method in our experience for detecting long-term virus replication in the sow. Protection from PRRSV-induced reproductive failure was defined as a lack of transplacental infection following virus challenge during late gestation determined by testing piglet blood collected at birth for PRRSV. The presence of PRRSV in the serum of at least one piglet in a litter indicated transplacental infection. Post-partum transfer of virus within a litter was determined by testing blood and lung lavages collected from each pig at the time of necropsy. Abortion was defined as expulsion of fetuses prior to GD 112.

A protective immune response in the sow was detected in 0 of 6 and 17 of 18 sows in groups A and B, respectively. Protection from PRRSV-induced reproductive failure was detected in 1 of 6 and 15 of 18 litters in groups A and B, respectively. Post-partum infection of pigs in group B was identified in 5 litters, 4 of which had no detectable congenitally infected pigs. This finding suggests the pigs may have been infected through contact with the sow, a hypothesis that is supported by the isolation of PRRSV from the colostrum sample of one non-immunized sow in group A.

Table 1. Virus isolation results for sera and lung lavage.

Sow	Sow						Pig and Fetuses ^c					
	I ^a	II ^{ob}	Necropsy				Parturition				Necropsy	
			GD ^c 90	GD 97	serum	Lung Lavage ^d	Live- born	Still- born	Late-term death	Mummy	serum	Lung Lavage
1	None	-	No	Yes	No	Yes	2/3 ^f	0	0/11	0	x	x
2	None	-	No	Yes	No	Yes	3/3	3/3	1/6	0	x	x ^g
3	None	-	No	No	No	Yes	0	0	2/3	0/4	x	x
4	None	-	No	Yes	No	Yes	0/6	0/3	0/1	0/5(0)	0/6	0/6
5	None	-	No	Yes	No	Yes	3/8	0	0/2	0	8/8	4/8
6	None	-	No	No	No	Yes	6/6	1/1	0/1	0/1	4/6	6/6
							14/26	4/7	3/24	0/10		
7	RespPRRS	147	No	No	No	No	0/12	0	0	0	0/11	7/11
8	RespPRRS	147	No	No	No	No	0/3	0	0	0	0/3	0/3
9	NADC-9(85)	142	No	No	No	No	0/10	0	0	0	0/10	0/10
10	NVSL-14(85)	168	No	No	No	No	0/11	0	0/4	0	0/11	0/11
11	NVSL-14(85)	168	No	No	No	No	0/12	0/1	0	0	0/12	0/12
12	NADC-8(85)	142	No	No	No	No	1/11	0	0	0/1	0/11	2/11
13	NADC-9(251)	138	No	No	No	No	0/6	0	0	0	0/6	1/6
14	NVSL-14(251)	162	No	Yes	No	No	0/11	0	0	0/1	1/11	3/11
15	NVSL-14(251)	138	No	No	No	No	0/14	0	0	0/1	0/13	0/13
16	NADC-8(251)	141	No	No	No	Yes	3/4	5/5	1/4	0/2	x	x
17	NADC-8(251)	141	No	No	No	No	0/10	0/2	0/2	0	0/6	3/6
18	NADC-9(wt)	152	No	No	No	No	0/17	0	0	0	0/16	0/16
19	NADC-9(wt)	166	No	No	No	No	0/12	0	0	0/1	0/11	0/11
20	NVSL-14(wt)	141	No	No	No	No	0/3	0	1/2	0/1	0/3	0/3
21	NADC-8(wt)	141	No	No	No	No	0/7	0	0	0	0/7	0/7
22	NADC-8(wt)	141	No	No	No	No	0/9	0/1	0	0	0/9	0/9
23	NVSL-14(wt)	138	No	No	No	No	0/9	0	0	0	x	x ^h
24	NADC-9(85)	168	No	No	No	No	0/13	0	0	0/2	x	x ⁱ
							4/174	5/10	2/12	0/9		

Key to Table 1.

^a Primary exposure to one of 4 strains of PRRSV. ^b Secondary exposure to a cocktail of 20 strains of PRRSV, interval in days between primary and secondary exposure. ^c Sow sera tested for virus on gestation day (GD) 90, 97 and at necropsy. ^d Lung lavage tested for virus at necropsy. ^e Virus isolation for sera and fluids collected from pigs and fetuses at birth and at necropsy. ^f Denominator = No. of pigs and fetuses; numerator = No. of pigs or fetuses from which PRRSV was isolated, x = pigs/fetuses all died at or soon after birth/abortion. ^g Sow aborted at GD 108, 18 days PE. ^h Sow aborted at GD 100, 10 days PE. ⁱ Sow had acute onset of rear limb paresis and was euthanized 10 days PE.

APPENDIX D: HOMOLOGOUS AND HETEROLOGOUS CHALLENGE OF PASSIVELY ACQUIRED PRRSV-SPECIFIC IMMUNITY IN PIGLETS

Three trials were conducted in this study utilizing pigs from 8 sows. All trials had a similar design in which pigs were bled at birth; at the time of weaning and virus challenge (6 to 7 days of age = day 0); and at weekly intervals after challenge. At weaning the pigs were divided into treatment groups and housed separately. Control group was oronasally exposed to sham inoculum. Virus challenge groups were oronasally exposed to either the NADC-8 isolate or the NADC-9 isolate of PRRSV.

Trial A -- The purpose of this trial was to evaluate the effect of passively acquired PRRSV-specific antibody on the replication of homologous and heterologous challenge virus in pigs. Twenty-three pigs derived from 2 dams infected with the NADC-8 isolate of PRRSV about 175 days before farrowing were divided into one of three groups: Control group that was not exposed to PRRSV; NADC-8 group that was exposed to homologous virus; and a NADC-9 group that was exposed to heterologous virus (a different virus isolate than what sows were exposed to previously). Piglets were monitored for 7 weeks PE and then euthanized at which time a lung lavage was collected and tested for PRRSV along with all sera samples collected from the pigs. Indirect fluorescent antibody tests were conducted on sera and reported as the geometric titer base 4. Results are reported in Table 1. One pig in the NADC-8 group was small and weak at weaning and became moribund 6 days post-weaning at which time the pig was euthanized. PRRSV was detected in the lung lavage but not in serum collected from this pig, data from this pig was not included in Table 1. PRRSV was not isolated from the lung lavage or sera collected from any other pigs in trial A. The antibody titer decreased overtime for each group suggesting the virus exposure at day 0 of age did not stimulate a detectable humoral immune response despite the possibility the some or all of the pigs may have replicated challenge virus based on the findings from the moribund homologous virus-challenged pig.

Trial B -- Since virus may have been replicating in pigs from trial A at an undetectable level by *in vitro* tests, trial B was designed with the objective of detecting homologous or heterologous challenge virus replication in pigs with passively acquired immunity by use of contact controls. Thirteen pigs derived from 2 dams infected with the NADC-8 isolate of PRRSV about 175 days

before farrowing were divided into one of three groups as previously described. Three days after virus challenge 5 pigs from 1 seronegative sow were used as contact controls for the virus-challenge groups; 2 pigs were placed into the NADC-8 group and 3 pigs into the NADC-9 group. Results are reported in Table 1. Contact control pigs in both virus challenge groups became infected with PRRSV as determined by seroconversion and the detection of virus in the lung lavage of each contact pig. Virus was not detected in the serum of any pigs in either virus challenge group although it was detected in lung lavage of each of these pigs at necropsy. The average antibody titer for each virus challenge group did not decline like the non-virus exposed control pigs in trial B or the 3 groups in trial A. Results from trial B suggest the contact-control pigs may have replicated virus and repeatedly exposed or reinfected the virus-challenged pigs in both groups resulting in a detectable immune response and replication of virus (Table 2).

Trial C -- The objective of trial C was to evaluate the incidence rate and temporal aspects of virus replication in the lung of seropositive virus-challenge pigs. Twenty-two pigs derived from 2 seropositive sows were divided into 3 challenge groups. Eleven pigs from 1 seronegative sow were divided into contact controls for all groups. Beginning at 1 week post challenge, virus-challenged pigs were necropsied with the following schedule: NADC-8 challenge group 2, 3, 2, and 0 pigs and the NADC-9 challenge group 2, 2, 2, and 1 pigs at weeks 1, 2, 3, and 4, respectively. PRRSV was detected at 1, 2, and 3 weeks PE in the NADC-8 challenge group in 2, 2, and 1 pig respectively (Table 2). Virus was not detected in the sera nor lung lavage of the NADC-8 group contact pigs. PRRSV was detected at 1, 2, 3, and 4 weeks PE in the NADC-9 challenge group in 2, 1, 1, and 1 pig respectively. Virus was detected only in the serum collected at necropsy from 1 of the NADC-9 group contact pigs (Table 2).

Table 1: Virus isolation and serology results for trials A, B, and C.

		Weeks post challenge ^a												
Trial ^b		# of pigs	Birth ^c										MΦ ^e	
				0	1	2	3	4	5	6	7			
A	Control	7	0 ^d	5.0	4.0	4.4	3.7	3.0	2.4	2.3	2.0	0		
	NADC-8	7	0	4.7	3.9	4.3	3.7	2.9	2.4	2.6	2.3	0		
	NADC-9	8	0	5.1	4.1	4.1	3.5	2.8	2.3	2.4	2.4	0		
B	Control	4	0	5.0	N.S. ^f	3.5	4.0	3.3	3.0	2.8		0		
	NADC-8	5	0	4.8	N.S.	4.0	4.0	4.8	5.4	5.6		5		
	Contact ^g	2	0	0	N.S.	0	0	5.0	6.0	6.0		2		
	NADC-9	4	0	5.0	N.S.	4.3	3.5	5.0	4.8	4.8		4		
	Contact ^g	3	0	0	N.S.	0	0	5.0	6.0	5.3		3		
C ^h		Birth		0	1		2		3		4			
	Control	8	0	8	4.5	8	4.3	8	4.6	8	3.9	8	3.0	0
	Contact ^g	4	0	4	0	4	0	4	0	4	0	4	0	0
	NADC-8	7	0	7	4.3	7	4.3	5	4.4	2	5.0	0		5
	Contact ^g	4	0	4	0	4	0	4	0	4	0	4	0	0
	NADC-9	7	0	7	4.4	7	4.3	5	4.4	3	4.0	1	5.0	4
	Contact ^g	3	0	3	0	3	0	3	0	3	0	3 ⁱ	0	3

Table 2: Virus isolation and serology results on individual pigs in trials B and C. ^a

TRIAL B										TRIAL C									
	pig#	Birth ^b	Weeks post challenge								pig#	Birth	Weeks post challenge						
			0	1	2	3	4	5	6				0	1	2	3	4		
NADC-8	27	0 ^c	5	N.S. ^d	3	3	4	5	(6) ^e	NADC-8	57	0	5	(5)					
NADC-8	28	0	4	N.S.	4	4	5	6	(6)	NADC-8	58	0	6	5	(6)				
NADC-8	29	0	5	N.S.	4	5	6	6	(6)	NADC-8	59	0	5	5	6	4			
NADC-8	30	0	4	N.S.	4	3	5	6	(6)	NADC-8	68	0	3	4	4	(6)			
NADC-8	35	0	6	N.S.	5	5	4	4	(4)	NADC-8	69	0	3	4	3				
Contact ^f	37	0	0	N.S.	0	0	5	6	(6)	NADC-8	70	0	4	3	(3)				
Contact ^f	38	0	0	N.S.	0	0	5	6	(6)	NADC-8	71	0	4	(4)					
										Contact	46	0	0	0	0	0	0	0	0
NADC-9	31	0	4	N.S.	4	3	5	5	(6)	Contact	47	0	0	0	0	0	0	0	0
NADC-9	32	0	5	N.S.	4	3	6	4	(5)	Contact	48	0	0	0	0	0	0	0	0
NADC-9	33	0	5	N.S.	4	3	5	6	(5)	Contact	49	0	0	0	0	0	0	0	0
NADC-9	36	0	6	N.S.	5	5	4	4	(3)										
Contact ^f	39	0	0	N.S.	0	0	5	6	(6)	NADC-9	60	0	4	(5)					
Contact ^f	40	0	0	N.S.	0	0	5	6	(5)	NADC-9	61	0	5	5	5				
Contact ^f	41	0	0	N.S.	0	1	5	6	(6)	NADC-9	62	0	4	4	5	(5)			
										NADC-9	63	0	4	5	6	5	(5)		
										NADC-9	72	0	5	4	3	3			
										NADC-9	73	0	5	3	(3)				
										NADC-9	74	0	4	(4)					
										Contact	50	0	0	0	0	0	(0)		
										Contact	51	0	0	0	0	0	(0)		
										Contact	52	0	0	0	0	0	(0)		

Key to Table 1.

^a Pigs weaned at 6-7 days of age (time = 0) and challenged with either sham (control), homologous virus (NADC-8) or heterologous virus (NADC-9). ^b Trial A, B, and C described in Appendix D. ^c Birth-samples are sera collected prior to suckling. ^d Group average PRRSV-specific antibody titer reported as arithmetic mean base 4. ^e Lung lavage collected at necropsy and tested for PRRSV, recorded as number positive for virus in group. ^f N. S. = Not Sampled. ^g Seronegative contact control pigs, see Appendix D for details. ^h For trial C data recorded as the number of pigs in group at time of sampling, number of pigs viremic, and average PRRSV-specific antibody titer for pigs within a group reported as arithmetic mean base 4. Beginning at 1 week post challenge, pigs were necropsied in both virus-challenge groups with the following schedule: NADC-8 challenge group 2, 3, 2, and 0 pigs and the NADC-9 challenge group 2, 2, 2, and 1 pigs at weeks 1, 2, 3, and 4, respectively. ⁱ One pig in the contact group for NADC-9 was viremic at necropsy.

Key to Table 2.

^a Pigs weaned at 6-7 days of age (time = 0) and challenged with either sham (control), homologous virus (NADC-8) or heterologous virus (NADC-9). ^b Birth-samples are sera collected prior to suckling. ^c 0 = Individual pig PRRSV-specific antibody titer reported as base 4. ^d N. S. = Not Sampled. ^e PRRSV detected in lung lavage at necropsy. ^f Seronegative contact control pigs.

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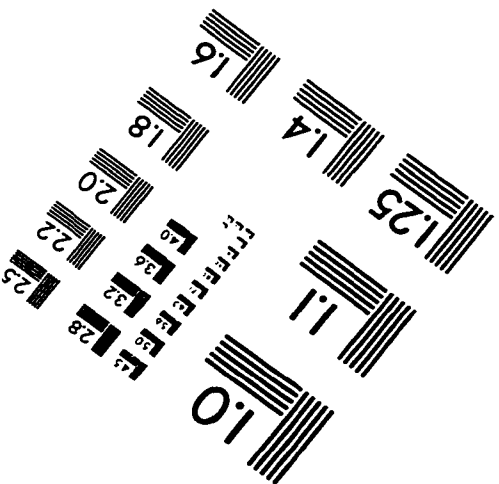
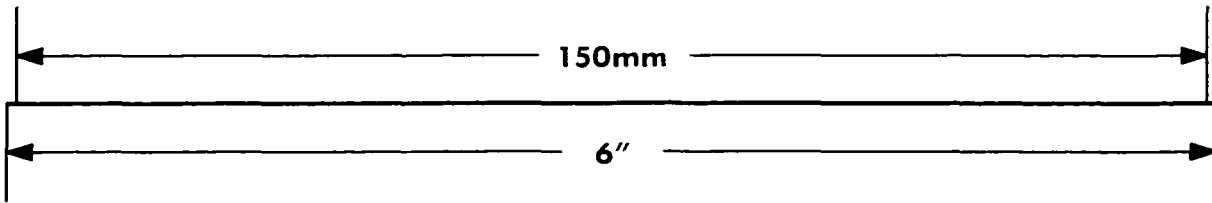
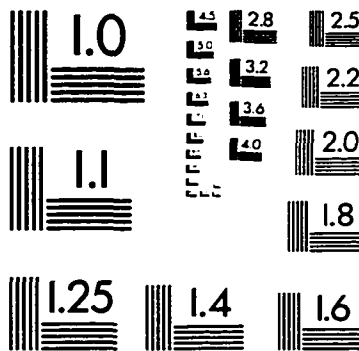
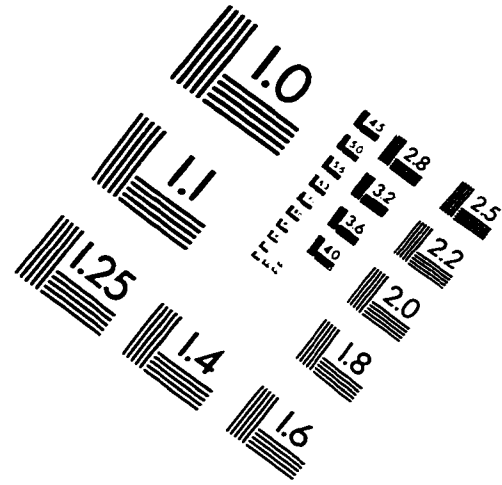
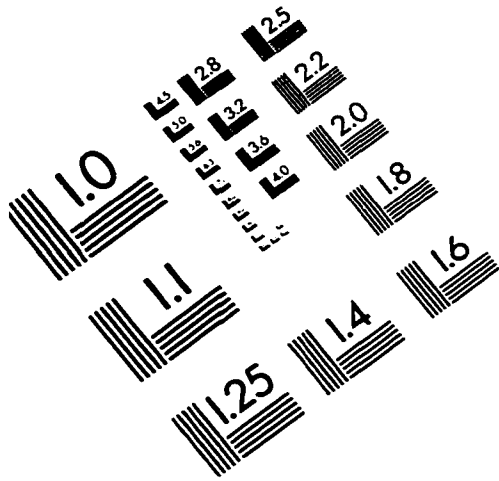
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IMAGE EVALUATION TEST TARGET (QA-3)



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